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THE BIOLOGICAL FUNCTION OF VITAMIN A ACID

JOHN E. DOWLING AND GEORGE WALD*

BIOLOGICAL LABORATORIES OF HARVARD UNIVERSITY, CAMBRIDGE

Communicated March 24, 1960

The first symptom of vitamin A deficiency in man and other animals is the rise of visual threshold known as night-blindness. This is also the only symptom the cause of which is well understood. The photosensitive pigments of the rods and cones (rhodopsin, iodopsin) upon which the visual threshold depends are formed from vitamin A ($C_{19}H_{27}CH_2OH$), through the combination of its aldehyde, retinene ($C_{19}H_{27}CHO$), with specific proteins of the rods and cones called opsins.[1] In rats maintained on a vitamin A-deficient diet, after initial stores of vitamin A in the liver and blood have been exhausted, the level of rhodopsin in the retina begins to fall, the logarithm of the visual threshold reciprocally rising, marking the beginning of night-blindness.[2]

Several weeks later, the level of opsin in the retina also begins to decline, and with this the rod outer segments deteriorate anatomically.[2] An explanation has been suggested for this effect also. Opsin in aqueous solution is a much less stable protein than rhodopsin. It is rapidly denatured by exposures to heat,[3] acids and alkalies[4] that leave rhodopsin intact. When, as the result of the deficient diet, the retina comes to contain opsin that can find no vitamin A with which to combine, this intrinsic instability is probably the cause of its disintegration; and since the outer segments of the rods are largely composed of this protein, as opsin is lost their anatomical structure must suffer accordingly.

The deterioration of opsin and of the rod outer segments, however, is only a detail in a much wider complex of changes occurring throughout the animal at the same time; for at this time all the overt signs of vitamin A deficiency appear: loss of weight, postural imbalance, respiratory disturbances, corneal opacities, disarrangement of coat, and red secretions about the eyes. Tissues, perhaps particularly epithelia, have begun to disintegrate in many parts of the body, and within a few days more all these animals are moribund.[2]

It has long been recognized that vitamin A plays some general role in the tissues, indispensable for their integrity and the growth and maintenance of the animal. The nature of this, by far its most important function, is as yet altogether unknown. Our experiments, having begun with well-understood processes in the retina, had at this point become involved with these wider and wholly obscure phenomena.

It seemed possible that we might be able to disentangle this situation, and perhaps learn something of the tissue function of vitamin A, with the help of vitamin

A acid. This substance ($C_{19}H_{27}COOH$), first prepared by Arens and Van Dorp,[5] was shown to maintain growth in the rat and to stave off obvious signs of deficiency, with a bipotency approaching that of vitamin A itself;[6, 7] yet no matter how large the amounts in which it was fed, no vitamin A was deposited in the liver.[8] The rat seems unable to reduce vitamin A acid to vitamin A; and this special circumstance led Moore[9] to suggest that though the tissue functions of vitamin A seem to be fulfilled by vitamin A acid, this substance might *not* be able to serve as precursor of the visual pigments, which need for their synthesis the alcohol and aldehyde. These considerations formed the starting-point of the present investigation.

Methods.—Most of our procedures were described in an earlier paper.[2] Male, weanling rats from the Harvard colony were raised on the standard U. S. P. vitamin A-deficient test diet, to which supplements were added as wanted. Techniques for evaluating liver and blood vitamin A, rhodopsin, and opsin in the eye, and recording electroretinograms were as described earlier.

Vitamin A and vitamin A acid, dissolved in cottonseed oil, were administered by mouth, through a syringe with a blunted point. Since in these experiments we were interested only in maximal effects of vitamin A acid, excessive doses were given. Three feedings a week provided a dosage level equivalent to at least 50 μgm per day. This level was chosen with the thought that if the acid possesses the lowest activity yet reported for it—10 per cent as high as vitamin A[6]—we should still be providing about twice the vitamin A-activity considered to be adequate for the rat (2–2.5 μgm vitamin A per day).

A Typical Experiment.—The course of these experiments and the general nature of the results can best be introduced with such a typical experiment as shown in Figure 1. Two animals, litter mates, were placed on the deficient diet, supplemented in one of them with vitamin A acid as described. Both animals grew at about the same rate for 5–6 weeks. Then the unsupplemented animal stopped growing, rapidly lost weight, and died on the 57th day of the diet. The other animal continued to grow regularly, and appeared to remain in prime condition, as the photograph taken on the 157th day of the diet is intended to show. On the same day this rat's electroretinograms were recorded, as shown at the right of Figure 1, compared with those of a normal animal measured at the same time.

This animal, though normal in weight and appearance, was highly night-blind. Its visual threshold—the luminance of a $^1/_{50}$-second flash needed to excite a just measurable ERG—was 3.25 log units (about 1,800 times) above normal. As the figure shows, this animal yielded about the same ERG at log luminance 4 as the normal animal did at log luminance 0. Rhodopsin could be extracted from the retinas of animals in this condition in only 1–5 per cent of normal amounts. This degree of night-blindness was higher than we had ever achieved before in rats kept on the vitamin A-deficient diet without supplementation.

On the following day these animals were sacrificed, and the retinal histology examined (Fig. 2). In the animal kept for 5 months on vitamin A acid, all the retinal tissues were normal in appearance, except for the visual cells. The pigment epithelium, bipolar layer (inner nuclei), and ganglion cell layer (not shown; see Fig. 15) did not seem in any way altered.

The nuclei of the visual cells (outer nuclei) were considerably reduced in num-

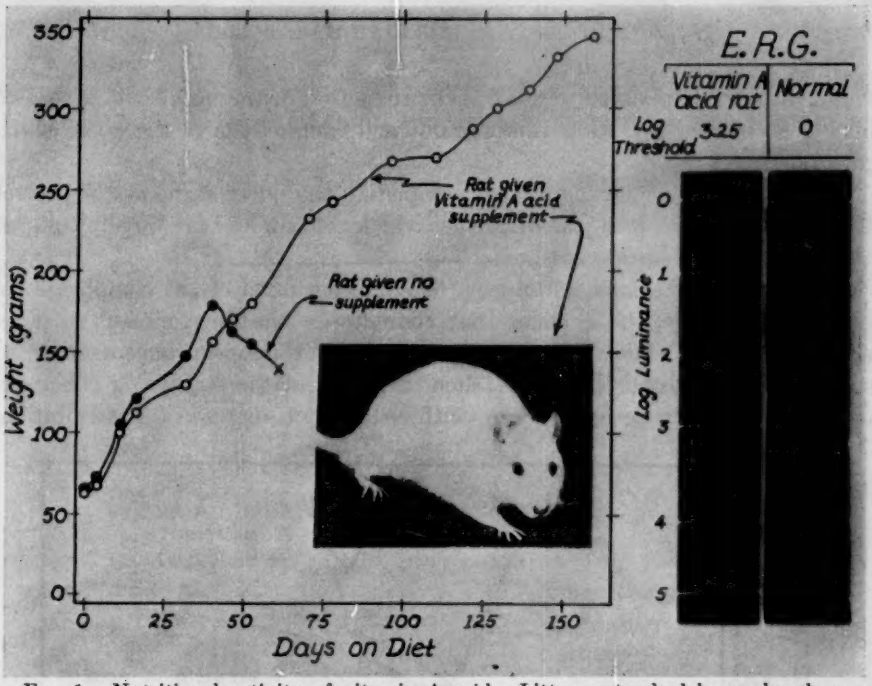


FIG. 1.—Nutritional activity of vitamin A acid. Litter mates had been placed on a vitamin A-deficient diet, and on the same diet supplemented with vitamin A acid. The rat given no supplement died on the 57th day of the diet; the animal receiving vitamin A acid continued to grow and remained in excellent condition for the duration of the experiment, a little over 5 months. The picture of this animal was taken at the end of the experiment, as were the electroretinograms shown at the right, compared with those of a normal animal. They show this rat to be highly night-blind: its visual threshold had risen 3.25 log units (about 1,800 times) above normal, and only just detectable ERG's could be evoked at even the highest luminances.

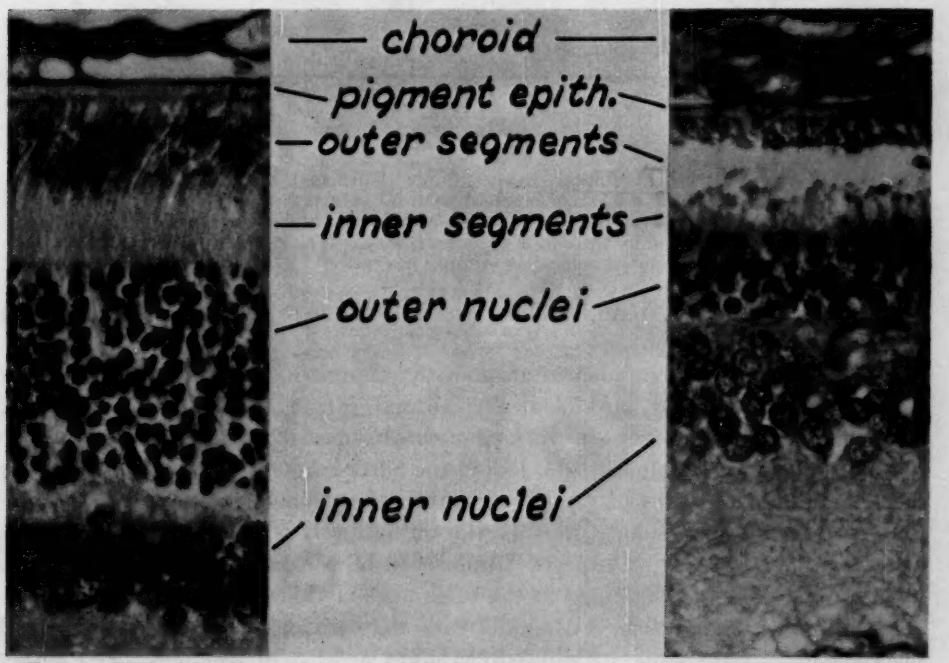


FIG. 2.—Retinal histology of the night-blind animal shown in Fig. 1, compared with that of a normal animal. All the retinal tissues are normal except the visual cells, which are reduced in number and almost completely lack outer segments.

ber, but otherwise appeared normal. The reduction in the number of visual cells probably accounted also for a thinning out and compression of the layer of inner segments.

This retina however lacked almost completely the outer segments of the rods. With the loss of rhodopsin, the organelles which contain and are largely composed of this pigment had almost vanished.

Such an animal displays, as an isolated condition freed of the complications of general tissue decay, the changes that characterize the development of dietary night-blindness: on removal of vitamin A, the fall in rhodopsin concentration, with associated rise of visual threshold; then, deprived of the stabilizing effect of its prosthetic group, the decay of opsin, with consequent anatomical deterioration of

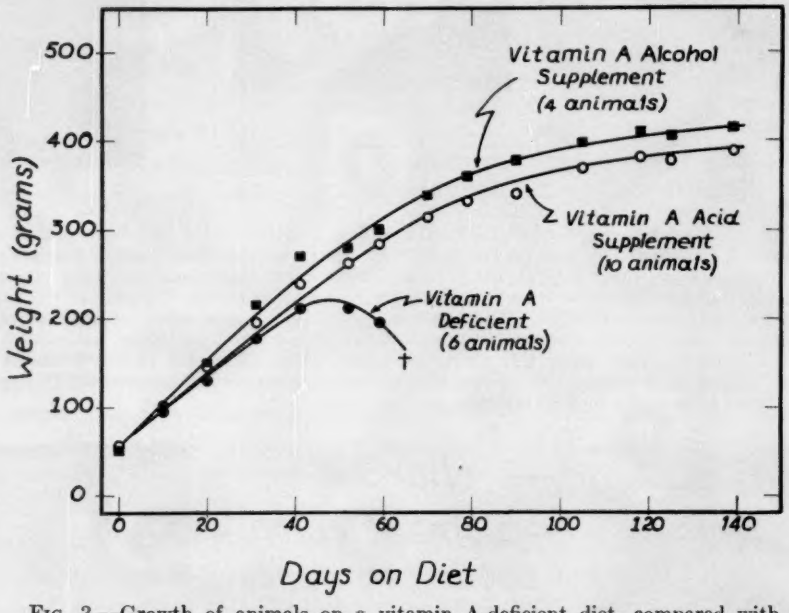


FIG. 3.—Growth of animals on a vitamin A-deficient diet, compared with growth on the same diet supplemented with 50 μgm per day of vitamin A or vitamin A acid. The unsupplemented animals lost weight after 6 weeks on the diet, and all had died by the end of the eighth week. The supplemented animals grew as well on vitamin A acid as on vitamin A.

the rod outer segments, in all likelihood the only tissue in the body of which opsin is an important component.

In this instance the supplementation with vitamin A acid had resulted in an animal which appeared physiologically normal except for its night-blindness; biochemically normal, except for its lack of rhodopsin and opsin; and anatomically normal, except for the almost total loss of the outer segments of the rods. By the same token, this animal seemed to demonstrate that the only function in the body that requires vitamin A itself may be the formation of visual pigments. All the general tissue functions of vitamin A appear to be performed by vitamin A acid. These conclusions are examined in the remainder of this paper.

Growth and Maintenance.—Weanling rats were divided into three groups, all placed simultaneously on the standard vitamin A-deficient test diet. One group

was supplemented with vitamin A, the second with vitamin A acid, both dissolved in cottonseed oil; and the third group was given the same amount of cottonseed oil alone.

The growth of these animals is shown in Figure 3. For the first 5 weeks on the diet, all three groups grew about equally. Then the unsupplemented animals

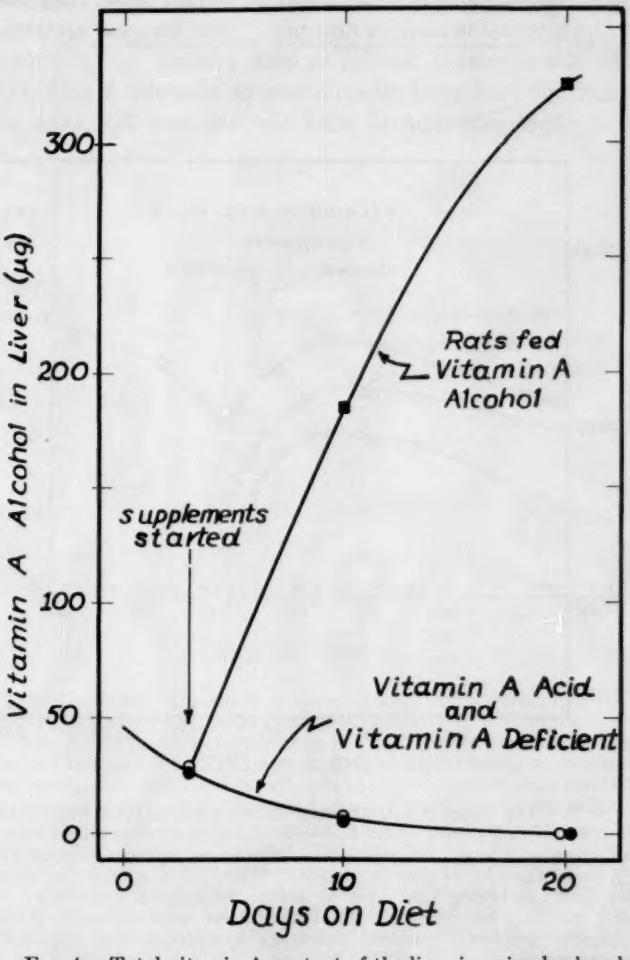


FIG. 4.—Total vitamin A content of the liver in animals placed on a vitamin A-deficient diet, and on the same diet supplemented with 50 μgm per day of vitamin A or vitamin A acid. (These animals formed part of the same experiment as in Fig. 3.) The animals supplemented with vitamin A rapidly increased their liver stores. Those receiving vitamin A acid lost their initial stores of vitamin A as rapidly as those receiving no supplementation.

stopped growing, declined rapidly in weight, and by the end of the eighth week, all had died. The animals receiving supplements of vitamin A and of vitamin A acid grew equally well throughout the experiment (140 days). The small difference in average weight shown in Figure 3 (27 grams; 6.7 per cent) does not appear to be significant. Each of the supplemented groups spread considerably in weight, and

overlapped each other widely (vitamin A: 360–450 grams; vitamin A acid: 318–512 grams). In another experiment, after 135 days of a similar regime, the average weight of the animals on vitamin A acid was slightly *greater* than that of the animals receiving vitamin A (324:314 grams). It may be concluded that the growth of the animals on vitamin A acid was entirely normal.

Both groups of animals also appeared equally sound, externally and in the gross appearance of the internal organs on autopsy. The tracheal epithelium, examined microscopically, also appeared normal in both groups.

To test further the biological effectiveness of vitamin A acid, this supplement was withheld in other experiments until the 4th and 7th week of the vitamin

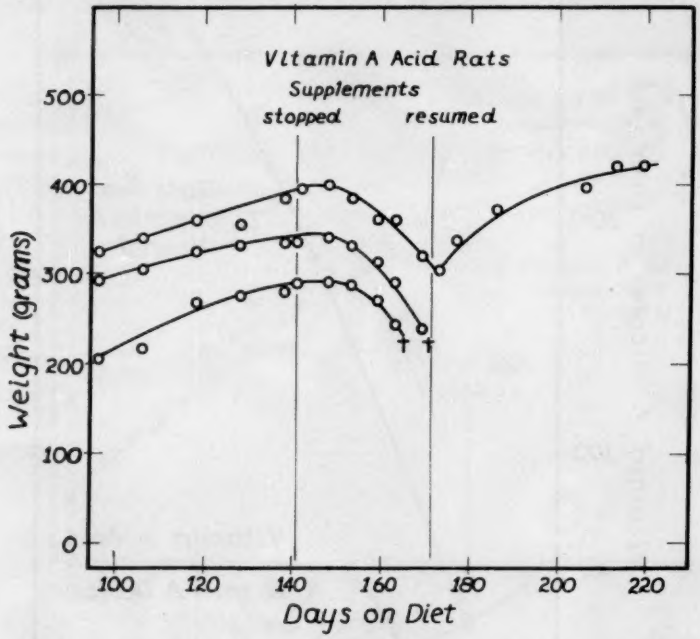


FIG. 5.—The effect of withdrawing the supplementation from animals growing normally on a vitamin A-deficient diet supplemented with excess vitamin A acid. Within a few days these animals stopped growing, and began to decline in weight. Within 3–5 weeks, two animals had died displaying the signs of severe vitamin A deficiency. The third animal, on renewed supplementation with vitamin A acid, promptly recovered from its deficiency symptoms, and regained its normal weight.

A-deficient diet, that is, until the animals had stopped gaining or were rapidly losing weight. On administration of vitamin A acid, such animals began at once to grow, and soon appeared identical with those supplemented from the start of the experiment. Also any signs of vitamin A deficiency that had developed on the deficient diet rapidly healed, except for occasional scarring of the cornea, owing to earlier xerophthalmia, which remained permanently.

Vitamin A Storage and Depletion.—We had noticed that night-blindness develops equally rapidly in rats on a vitamin A-deficient diet, whether or not supplemented with vitamin A acid. During the first weeks on the diet such animals use up the supplies of vitamin A stored in the liver; and it seemed from this observation that

the rate of depletion of vitamin A in the liver is probably equally rapid, whether or not vitamin A acid is available.

Figure 4 shows this to be the case. The vitamin A content of the liver was measured in animals divided into the three dietary groups already described; indeed these animals formed part of the same experiment shown in Figure 3. Those receiving supplements of vitamin A (50 μgm per day) rapidly increased their stores

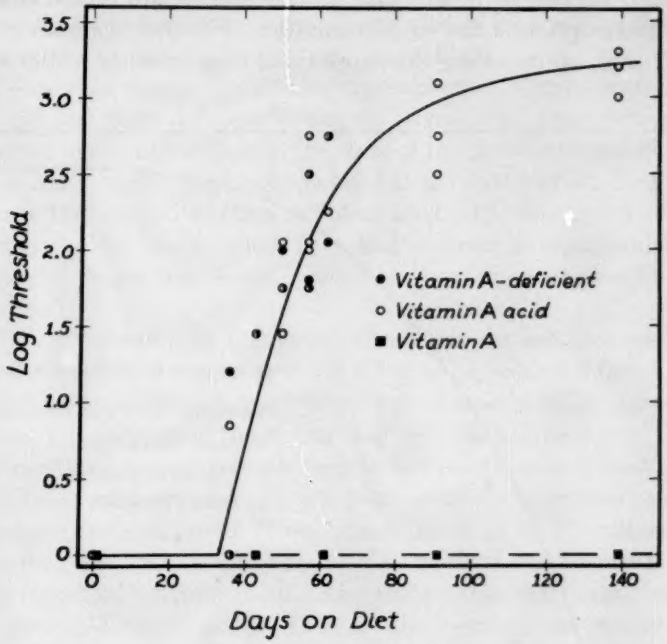


FIG. 6.—Visual thresholds of animals kept on a vitamin A-deficient diet, and on the same diet supplemented with vitamin A or vitamin A acid (same experiment as in Fig. 3). The threshold is the smallest luminance of a $^1/_{20}$ second flash of light needed to evoke a just-perceptible ERG. The vitamin A-supplemented rats formed the control group, whose thresholds are arbitrarily given the value 1 (log threshold = 0). All other thresholds are expressed relative to these, and represent therefore increments of log threshold above the control level. In the animals supplemented with vitamin A acid, the threshold rises as soon and as rapidly as in those receiving no supplementation. All the latter group have died, however, by the end of the ninth week; whereas the vitamin A acid animals survive to grow more night-blind. The thresholds level off after 12–15 weeks on the diet, at about 3.25 log units (about 1,800 times) above normal. At this time the retinas contain only 1–5 per cent of the normal amounts of rhodopsin.

in the liver. Those supplemented with vitamin A acid, however, lost their initial stores of liver vitamin A as rapidly as the rats receiving no supplements. Both groups also developed night-blindness at the same time. Vitamin A acid not only fails to contribute vitamin A to the liver, but seems to have no sparing action on the vitamin A already there. Since, however, the animals receiving the acid supplement are adequately maintained by it, except for their vision, the loss of vitamin A from the liver seems to be independent of demand. We shall have more to say of this relationship below.

The animals supplemented with vitamin A acid fail also to store this substance. Vitamin A acid is readily identified by the sharp absorption band at 573 mμ which it yields in the antimony chloride test. We have never by this means been able to detect the acid in extracts of liver, kidney, or blood of animals receiving large amounts of this substance in the diet (cf. also Sharman[7]).

One consequence of the failure to store either vitamin A or the acid is that these animals, though normal in weight and in excellent health, respond almost immediately to interruption of the supplementation (Fig. 5). Animals growing well on vitamin A acid, on removing the supplement stop growing within a few days,

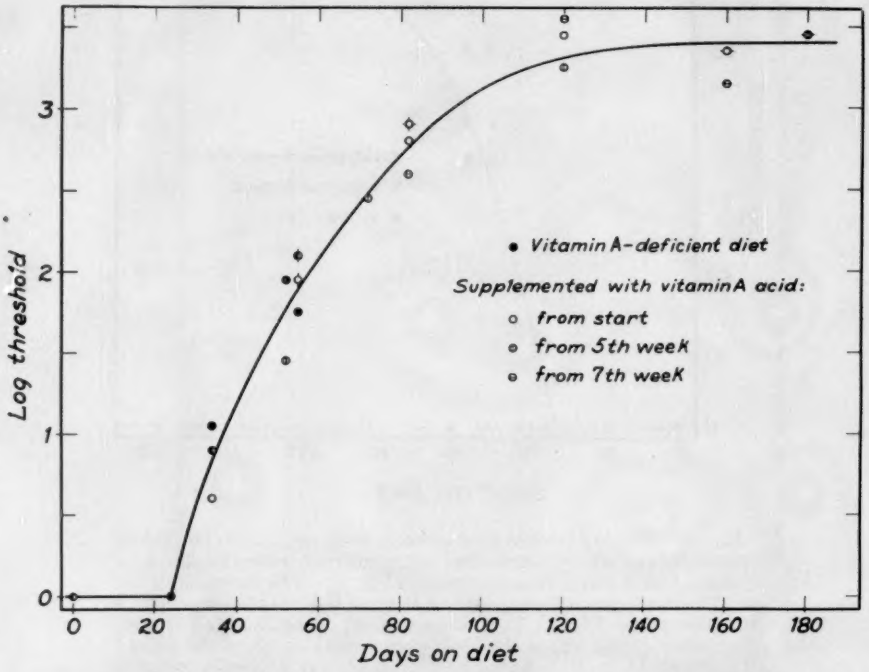


FIG. 7.—Development of night-blindness in animals on a vitamin A-deficient diet, and on the same diet supplemented with vitamin A acid from the start, or from the 5th week—when the animals had stopped growing—or from the 7th week, when they were declining rapidly in weight. In all these animals the visual threshold begins to rise at the same time, and rises equally rapidly. By the end of the 8th week, the unsupplemented animals have died; whereas those receiving vitamin A acid grow increasingly night-blind. After about 18 weeks on the diet the visual threshold of the latter group becomes constant at about 3.3 log units (about 2,000 times) above the normal level.

decline rapidly in weight, and within 3–5 weeks die displaying all signs of severe vitamin A deficiency. That is, these animals respond as do young animals on a vitamin A-deficient diet after all internal stores of vitamin A have been exhausted. If the supplementation with vitamin A acid is renewed, even toward terminal stages of the deficiency, such animals rapidly recover (Fig. 5).

Night-Blindness.—We have already remarked that animals supplemented with vitamin A acid, though otherwise sound, become night-blind as rapidly as those receiving no supplementation. This is shown in Figures 6 and 7.

Figure 6 shows the visual thresholds—the brightness of a $^1/_{50}$ second flash of light required to excite a just perceptible electroretinogram—of the animals whose weights are shown in Figure 3. Those receiving supplements of vitamin A served as controls; their thresholds are arbitrarily given the value 1 (log threshold = 0), and all other thresholds are expressed as increments of log threshold above these control values. It is clear that the threshold rises at the same time and at about the same rate in the animals receiving no supplementation, and those receiving vitamin A acid; but whereas the former have all died by the 60th day of the diet, the latter survive to become increasingly night-blind.

Figure 7 shows the results of a similar experiment, conducted with more animals and hence yielding more accurate averages. Again the unsupplemented animals and those given vitamin A acid from the start of the diet developed night-blindness at the same rate. Again the unsupplemented animals had all died by the 60th day; whereas the others, whether supplemented with vitamin A acid from the start, or from the 5th week—when they had stopped growing—or from the 7th week—when the weight was declining rapidly—continued to grow and to become more night-blind.

In both these experiments the visual thresholds do not increase indefinitely, but level off after 12–15 weeks at 3.3–3.5 log units—about 2,000–3,000 times—above normal. Direct extraction of the retinas of such animals showed that they contain about 1–5 per cent of the normal amounts of rhodopsin.

The electroretinograms of animals maintained on vitamin A acid are shown in Figure 8. The stimulus was a $^1/_{50}$ second flash of light, the luminance of which was varied in steps over a range of 7 logarithmic units (1 to 10 million). After 28 days on the diet, the ERG is still normal. At 56 days, the animal is quite night-blind. The threshold has risen about 2.8 log units (about 500 times); at luminances above the threshold the response—both a- and b-waves—is greatly diminished; and a small inflection normally found on the downward sweep of the b-wave has separated off as a second positive wave. All these effects are characteristic of this stage of night-blindness in unsupplemented, vitamin A-deficient animals.[2]

As the animals continue on vitamin A acid supplementation, the electroretinograms undergo a second type of change. The threshold, as already described, becomes relatively constant by about the 120th day of the diet, at about 3.2–3.5 log units above normal. Increasing the intensity of the stimulus above the threshold level, however, now begins to have less and less effect. At 139 days, stimuli even 4 log units above threshold yield only a slow and diffuse b-wave of low amplitude; indeed by that time the intensity of the stimulus hardly affects the form or height of the response (Fig. 8). It is as though, following upon the rise of threshold that marked the first stage of night-blindness, these animals now lose the capacity to generate an ERG. As Figure 8 shows, after 10 months on the regime, no response can be elicited at all.

Figure 9 shows the relation between the visual threshold and the quantity of rhodopsin extracted from the retina. We had found earlier that in vitamin A-deficient animals receiving no supplementation, the *logarithm* of the threshold rises linearly as the rhodopsin content falls.[2] In animals supplemented with vitamin A acid, this relationship can be extended further. As Figure 9 shows, it is maintained over the whole range of the measurements.

Figure 9 involves a further comparison. The rhodopsin content of the retina falls in vitamin A deficiency; it falls also on exposure of the animals to bright light (light adaptation), rising again when the light is extinguished. That is, vitamin A deficiency and light adaptation are two ways of inducing night-blindness. How does the relationship between rhodopsin content and log threshold compare in both conditions? To answer this question a group of normal rats was highly light-

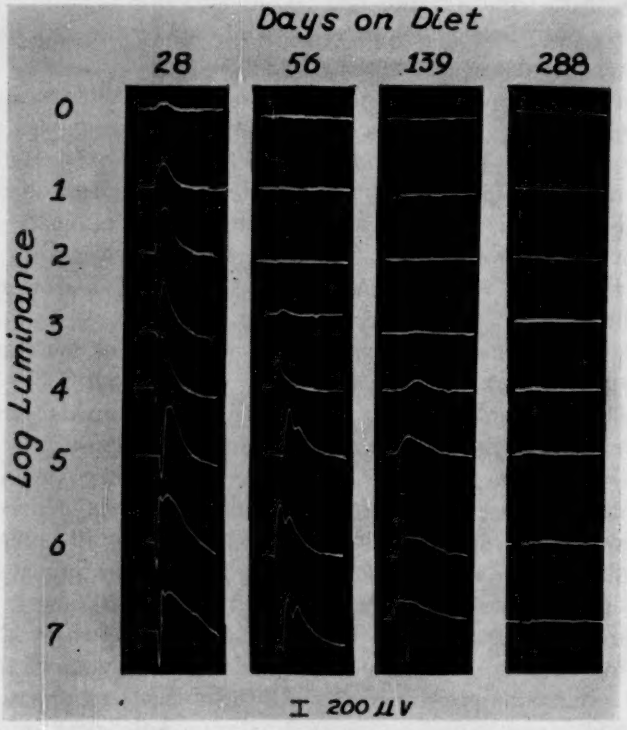


FIG. 8.—Electroretinograms of animals on a vitamin A-deficient diet supplemented with vitamin A acid. Responses to a 1/50 second flash of light, at luminances ranging over 7 log units (1 to 10 million). The initial deflection downward is the negative a-wave; the later sweep upward is the positive b-wave. The first strip of records was made on the 28th day of the diet, when the ERG is still normal. On the 56th day the animal is typically night-blind: its threshold has risen about 500 times; the a- and b-waves have decreased greatly in amplitude; and a small inflection on the downward sweep of the b-wave has separated off as a second positive wave. By the 139th day, the threshold has risen only moderately further; but now increasing the brightness of stimulus above the threshold level has little effect. The retina is losing the capacity to generate an ERG. By the 288th day (10 months on the diet), no response can be elicited at the highest available brightness. The animal is now blind.

adapted; then the light was shut off, and periodically an animal was anaesthetized and its ERG threshold determined. The same, and also other groups of animals were light-adapted similarly, and after the same intervals in darkness animals were killed, and their retinas extracted for rhodopsin. In this way the relationship between visual threshold and the retinal content of rhodopsin was measured, this time however in normal animals in the ordinary course of dark adaptation.[10, 11]

Figure 9 shows that a change of rhodopsin concentration in the retina, whether induced by visual adaptation or by vitamin A deficiency, has the same effect on the threshold. In both conditions the logarithm of the threshold rises linearly as the rhodopsin content falls.[12] The identity of these effects implies that up to this point in the development of vitamin A deficiency, the night-blindness is accounted for completely by the loss of rhodopsin from the retina.

Anatomical Changes.—We have already seen that by the 24th week on vitamin A acid, the visual cells are considerably reduced in number, and most of the rods lack outer segments (Fig. 2). The disintegration of the rod outer segments seems

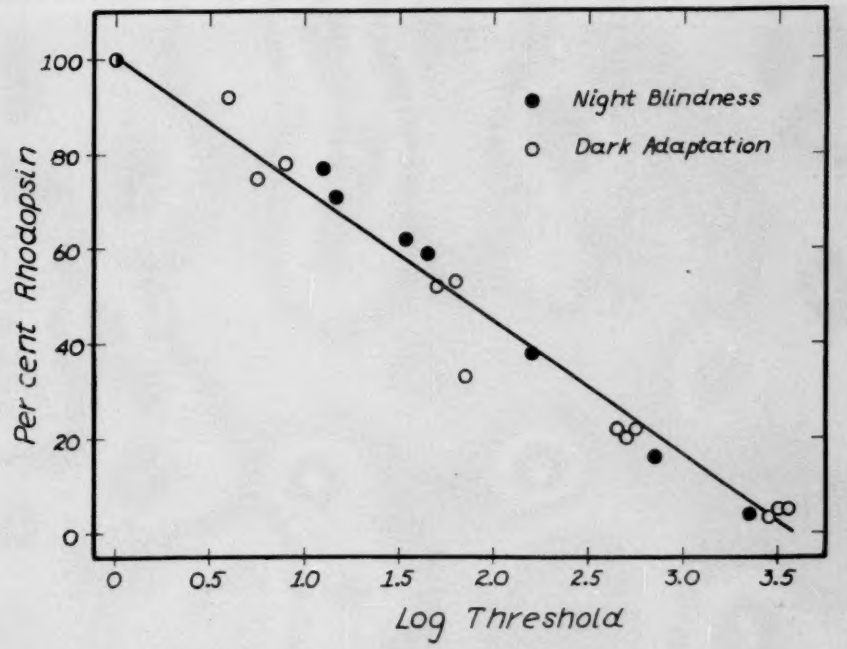


FIG. 9.—Relation between the rhodopsin content of the rat retina and the visual threshold, in animals night-blind owing to vitamin A deficiency, and in normal animals dark adapting after exposure to bright light. In both instances the same relationship is observed: the log threshold rises linearly (i. e., the log sensitivity—log 1/threshold—falls linearly) with fall in rhodopsin concentration. This relationship is described by the equation, $\log (I_t/I_o) = 3.6(R_o - R_t)/R_o$, in which I_o and R_o are respectively the threshold and rhodopsin concentration in dark adapted control animals, and I_t and R_t are respectively the thresholds and rhodopsir concentrations in vitamin A-deficient or partly dark-adapted animals.

to accompany the loss of opsin. For example, in one experiment, in the 18th week of the deficiency (with vitamin A acid supplementation), when the threshold had risen over 3 log units, and the rhodopsin level was only a few per cent of normal, the opsin had declined to half the normal level.

As the retina loses opsin, examination in the electron microscope shows progressive deterioration of the microstructure of the rod outer segments. The electron microscopy was performed in collaboration with Dr. I. R. Gibbons, and will be reported in detail elsewhere.[13] After removal of the cornea and lens, the whole fundus of the eye was fixed for one hour in buffered osmium tetroxide, washed, de-

hydrated with acetone, and embedded in araldite resin. Ultrathin sections were cut on a Porter-Blum microtome, and examined in the R. C. A. EMU-3D electron microscope.

Figure 10 shows a longitudinal section through portions of the rod outer segments of a normal rat. As in other animals, the outer segment consists of a stack of flattened sacs, wholly enclosed within an outer membrane. The outer segment is about 1.5 μ wide and about 15 μ long. It contains about 35 disks per μ, or about 525 disks in all.

Figure 11 shows an early stage in the deterioration of this structure, typical of animals kept 3–4 months on the vitamin A-deficient diet supplemented with vitamin

FIG. 10.—Electron micrograph of a longitudinal section through a rod outer segment, and portions of two neighboring outer segments, in the normal rat. As in other animals, the outer segment consists of a stack of transverse, flattened sacs, entirely enclosed in an outer membrane. The rat outer segment is about 1.5 μ wide and 15 μ long, and contains about 525 disks. Magnification 73,000.

A acid. Many of the retinal disks are still intact; but many others have segmented, or are in process of doing so, into groups of distended vesicles and tubules.

By 6 months on the regime, these changes have progressed further (Fig. 12). Few outer segments remain, and their internal structure is highly disorganized. They have lost also their characteristic cylindrical shape, becoming distorted and tending to collapse, often into roughly spherical shapes. By this time also the visual cells are considerably reduced in number, and their inner segments, formerly long and slender, have shortened and broadened (Fig. 2). The ultrastructure of the nuclei and inner segments of the visual cells, however, has not visibly changed.

As animals continue longer on this regime, the visual cells undergo further altera-

tions. Figure 13 shows sections of the retinas of two litter mates, one supplemented with vitamin A, the other with vitamin A acid, for 10 months on the otherwise A-deficient diet. The animal receiving vitamin A acid no longer yielded an ERG in response to the brightest lights available in our apparatus (Fig. 8). In this retina, the pigment epithelium and the layers of bipolar and ganglion cells still look normal. The visual cells, however, are reduced to a single row of nuclei, and the individual cells have contracted to a cuboidal shape, almost wholly occupied by the nucleus, with no distinguishable inner or outer segment. In this extraordinary state the pigment epithelium lies in direct contact with the layer of visual cell nuclei.

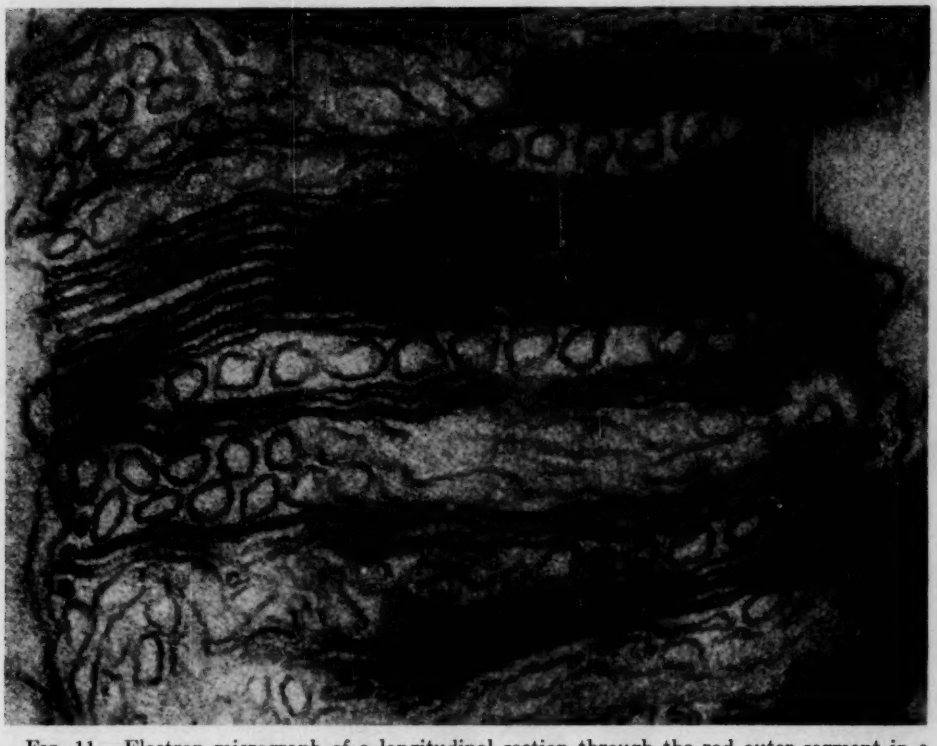


FIG. 11.—Electron micrograph of a longitudinal section through the rod outer segment in a highly night-blind rat, typical of animals that had been 3–4 months on a vitamin A-deficiency diet supplemented with vitamin A acid. Some of the transverse disks appear intact; others have segmented into groups of distended vesicles and tubules. Magnification 113,000.

Recovery.—On administration of vitamin A, animals which had become night-blind on the vitamin A-deficient diet supplemented with vitamin A acid recover in varying degree. When such animals have been on the diet up to 10 weeks, recovery ordinarily is rapid and complete. Within several hours after feeding a large dose of vitamin A in cottonseed oil, the visual threshold—which may by then have risen about 2 log units above normal—begins to fall, and within 2–3 days has reached the normal level.

After longer periods on the diet, recovery follows a more complex course. Figure 14 shows the effect of one intraperitoneal injection of 1 mgm of vitamin A on two animals that had been on the diet for 25 weeks. These animals recover in two

stages: the visual threshold first falls rapidly for 40–50 hours following the injection, reaching a level about 1 log unit above normal; thereafter it continues to fall very slowly over a period of many weeks of further vitamin A supplementation.

In animals kept still longer on the diet, these two phases of recovery change in relative importance, the fast phase contributing less, and the slow phase accounting for more and more of the total change.

It seems probable that these two stages of recovery are concerned with reversing the two phases we have described in the development of night-blindness: first, the rise of visual threshold associated with the decline of rhodopsin concentration owing

FIG. 12.—Electron micrograph of the longitudinal section through a rod outer segment in an animal that had been 6 months on the vitamin A-deficient diet supplemented with vitamin A acid. Few rod outer segments remain in such a retina; and, as in this example, they are highly distorted in shape and microstructure. A few isolated groups of disks still appear, but mainly the internal structure has degenerated into distended tubules and vesicles. The outer segments have also lost their normal cylindrical shape, and collapsed to irregular ellipsoids or spheres. Magnification 76,000.

to simple lack of vitamin A; later, the loss of opsin and anatomical deterioration of the rods. It seems reasonable to suppose that the fast phase of recovery involves the combination of whatever opsin remains with the vitamin A that is administered; whereas the synthesis of opsin and structural repair of the visual cells occupy the slow phase. Certain instances in which the recovery from experimental human night-blindness has exhibited similar fast and slow phases may perhaps be explained in the same way.[14]

In animals kept longer than 6 months on the diet, the visual threshold no longer recovers completely, even after months of vitamin A supplementation. This ir-

reversible aspect of night-blindness is probably associated with the actual loss of visual cells. Such cells, once lost, are never regained.

A decline in the density of visual cells must raise the visual threshold, just as a decrease in the area of the visual field normally raises the threshold; and this should be a source of various degrees of permanent night-blindness. If indeed it is true that a decrease in the density of visual cells has about the same effect as a decrease in the area of the visual field, it should raise the threshold by a factor corresponding to the reciprocal of this decrease (Riccò's Law) or the square root of the reciprocal (Piper's Law). That is, in such a condition as shown in Figure 2,

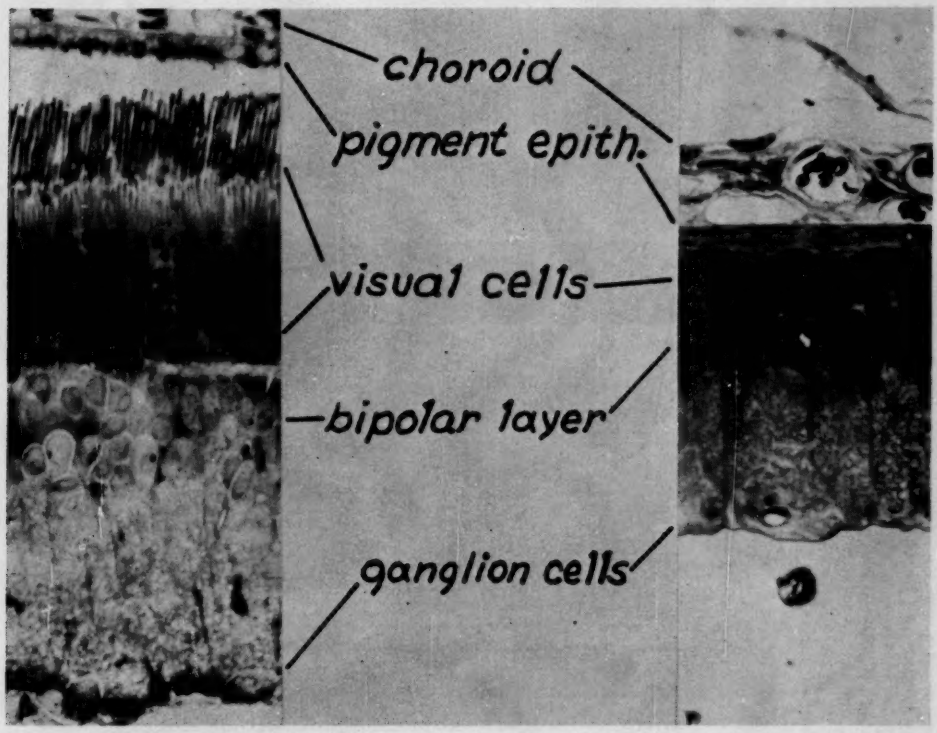


FIG. 13.—Sections through the retinas of two animals—litter mates—both of which had been 10 months on the vitamin A-deficient diet, supplemented with vitamin A (left), or with vitamin A acid (right). The retina on the left is normal; that on the right is also normal, except for its visual cells. The visual cells, only about $^1/_{10}$ of which remain, form a single, almost complete row of nuclei, directly apposed to the pigment epithelium. No inner or outer segments are visible. Such an animal is not only night-blind, but blind (cf. Fig. 8, ERG's at 288 days); and does not regain its vision after months of vitamin A supplementation.

in which the number of visual cell nuclei has been reduced to about $^4/_9$, if as a result of vitamin A administration all the remaining cells again became fully functional, without increasing in number, the final threshold should be raised permanently by $^9/_4$ or $\sqrt{^9/_4}$ (i.e., 2.25 or 1.50 times) over the normal threshold. Such changes do not loom large on a logarithmic scale: they correspond to raising the threshold 0.35 or 0.18 log unit.

A typical example of such an experiment is shown in Figure 15. Three litter mates were kept on the vitamin A-deficient diet for $6^1/_2$ months, one supplemented with vitamin A to serve as control, the other two supplemented with vitamin A

acid. Then the ERG's of the control and one vitamin A acid animal were measured; and on the following day the control and the other vitamin A acid animal were killed and their retinas sectioned.

The retina and ERG's of the control animal were normal (Fig. 15, left). The vitamin A acid animals, however, displayed the usual anatomical and physiological signs (Fig. 15, center). In the retina, only remnants of outer segments re-

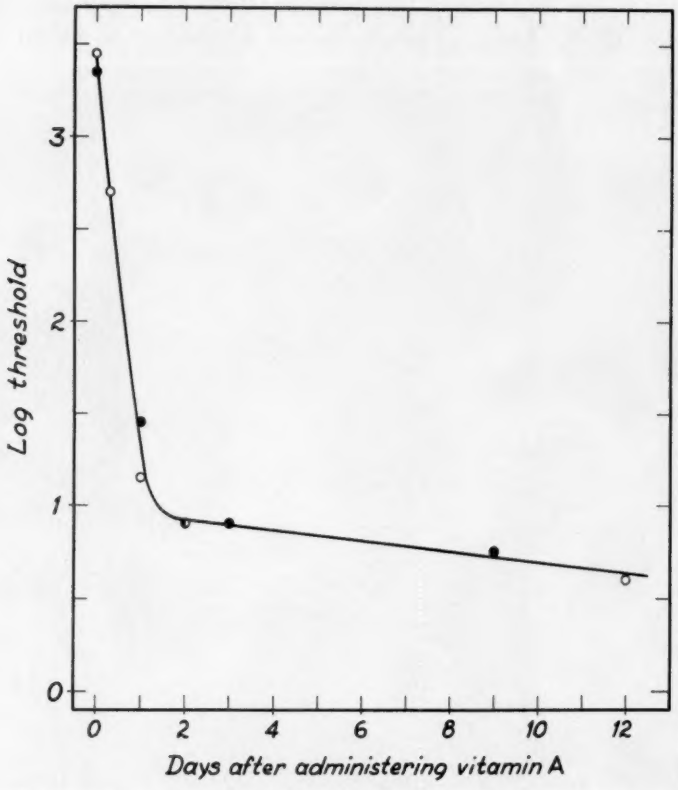


FIG. 14.—Recovery from night-blindness on administration of vitamin A. Two animals that had been kept for 25 weeks on the vitamin A-deficient diet supplemented with vitamin A acid received an intraperitoneal injection of 1 mgm vitamin A in cottonseed oil, followed by regular dietary supplementation with vitamin A. The visual threshold, starting from levels 3.3–3.4 log units (about 2,000 times) above normal, first falls rapidly to a level about 1 log unit above normal. Thereafter it continues to fall slowly for several weeks, returning almost to the normal level (cf. Fig. 15).

mained, and the inner segments were few and stumpy in appearance. The layer of visual cell nuclei was reduced from the normal 9 to about 4 rows. The ERG record shows the mate of this animal to have been highly night-blind; the visual threshold had risen more than 3 log units, and the ERG's at higher luminances displayed all the usual changes that go with this condition.

The surviving vitamin A acid animal was supplemented for 16 days with vitamin A. Then his ERG's were re-measured, and the retinas sectioned immediately

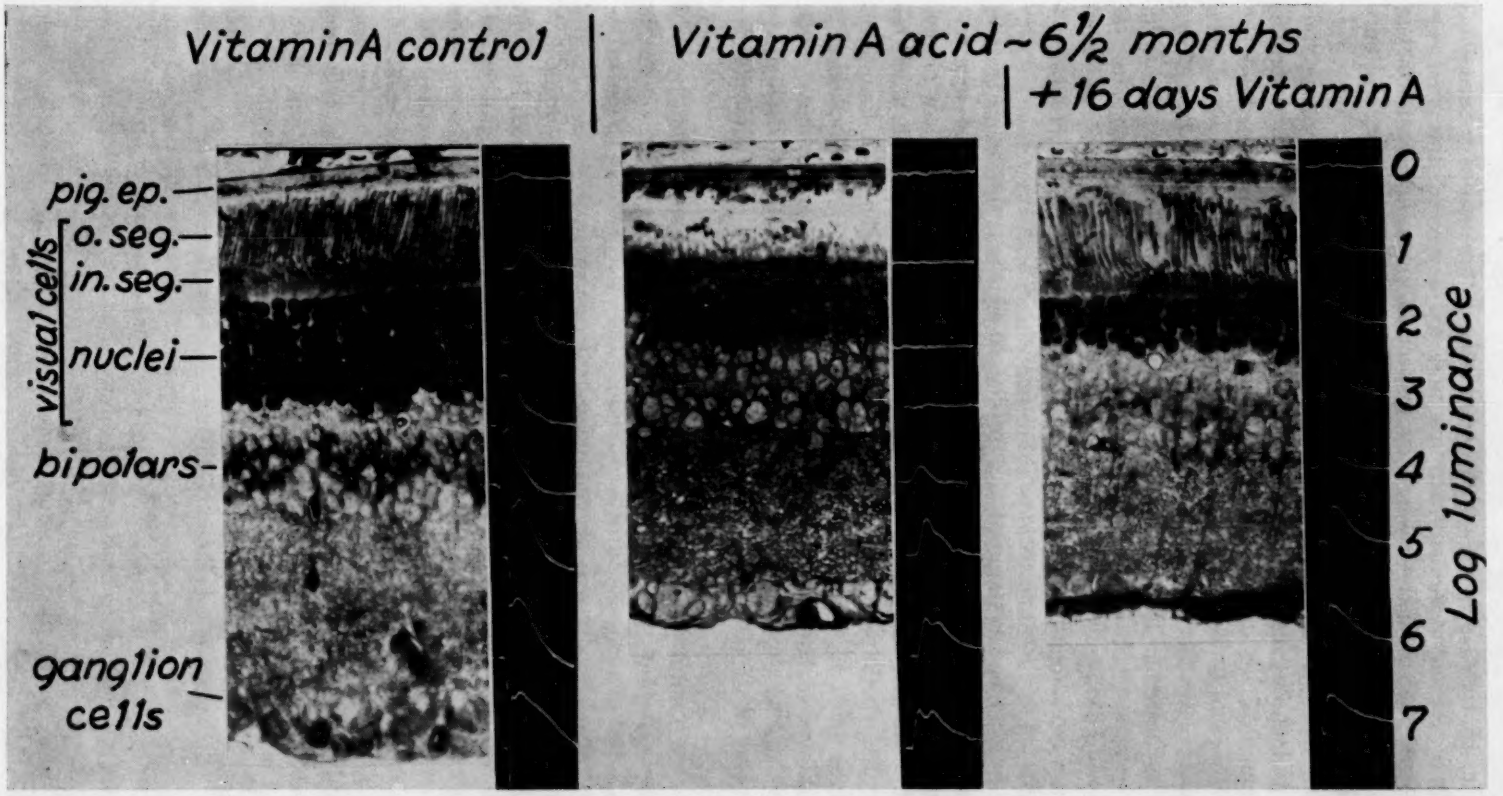


FIG. 15.—Development of and recovery from night-blindness. Three litter mates had been kept for $6^1/_2$ months on a vitamin A-deficient diet, supplemented in one with vitamin A to serve as control, and in the others with vitamin A acid. The ERG responses and retinal histology of the control animal are shown at the left. At this time the ERG's of one of the vitamin A acid animals were also measured; the other one was killed and its retinas sectioned (middle). The ERG's show a high degree of night-blindness, and the retina the almost complete loss of outer segments and reduction of the number of visual cells to about $^4/_9$ the normal population (compare Figs. 1 and 2). The surviving vitamin A acid animal was now given vitamin A supplementation for 16 days. Then its ERG's were re-measured, and its retinas sectioned immediately afterward (right). The rods are still reduced to about $^4/_9$ the normal number, but seem to have recovered their normal structures. The visual threshold has returned to about 0.25 log unit above the normal level; but the ERG's at higher luminances are much reduced in amplitude compared with the control. This small elevation of visual threshold above normal and decrease in the size of the ERG are apparently permanent effects, owing to the reduction in number and density of visual cells.

afterward (Fig. 15, right). In the retina, the visual cells seem to have been completely repaired: the outer segments are long and well-developed, the inner segments are shorter and broader than in a control retina, but otherwise normal in appearance. The visual cells, however, had not increased in number, and only about $^4/_9$ the normal population remained.

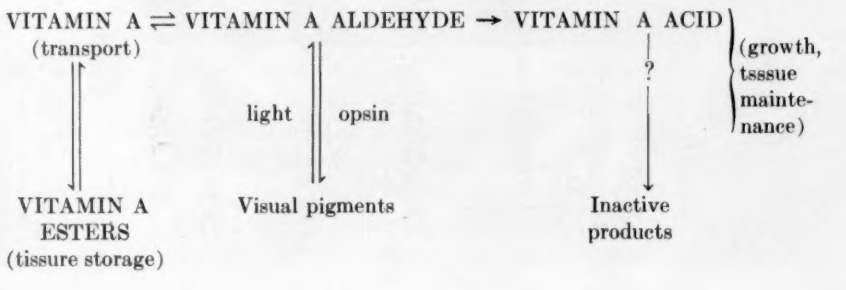
Under these circumstances the electroretinograms present an interesting condition. As we had anticipated, the visual threshold, though again nearly normal, remained about 0.25 log unit above that of the control litter mate—about the elevation of threshold to be expected with this reduction in number of visual cells. At higher levels of stimulation, the electroretinograms displayed a further effect: though once again normal in shape, they were considerably reduced in amplitude, presumably because the ERG's in such an animal are generated by a greatly reduced density of visual cells. Probably one could mimic all these effects in a normal animal by reducing the area of the visual field, and hence the numbers of visual cells affected by the stimulus.

An animal kept as long as 10 months on the diet, in which, as shown in Figure 8, even our brightest stimuli fail to evoke an ERG, displays no perceptible recovery even after months of high supplementation with vitamin A. Such an animal may be permanently blind. Yet it still possesses visual cells, though much reduced in number—perhaps still such a row of visual cell nuclei as in Figure 13. Why then does it fail to recover measurable visual responses? Such visual cells as shown in Figure 13 apparently can no longer regenerate outer segments, probably because by this time they have lost the flagellar structures—basal body and rudimentary shaft—from which the outer segments are embryonically derived.[15]

Discussion and Conclusion.—It appears from these experiments that the only function vitamin A may perform directly in the rat is to supply the prosthetic group of its visual pigments. All other functions—growth, general tissue maintenance—are served equally well by vitamin A acid.

The rat, however, seems unable to reduce the acid to the aldehyde or alcohol, as shown by the observation that no matter how high the level at which the acid is fed, no vitamin A is deposited in the liver, nor is any visual pigment synthesized. For this reason animals kept on a vitamin A-deficient diet supplemented with the acid, though they grow normally and remain in excellent health, become night-blind, indeed more night-blind than they survive to become on a vitamin A-deficient diet alone.

The general metabolism of vitamin A seems therefore to involve the following relationships (cf. ref. 9):



Vitamin A is commonly stored in the tissues, principally in the liver, as esters. It is transported in the blood mainly as the free alcohol.[16] The equilibrium of the alcohol dehydrogenase system which oxidizes it to retinene lies far over toward reduction;[17] no appreciable oxidation to retinene occurs unless the latter is removed as fast as formed. In the retina this is accomplished through trapping of the retinene by opsin to form the visual pigments.[18] The irreversible removal of retinene through oxidation to vitamin A acid may provide a second such mechanism.

The general growth and tissue functions of vitamin A appear to be fulfilled either by vitamin A acid itself (presumably in some combined or activated form) or by some product of its further metabolism. Experiments are in progress to determine what the active principle may be. It is already clear, however, that neither vitamin A acid itself, nor any derivative of it that may possess biological activity, is stored by the animal. Shortly after feeding the acid, none of it can be found in the blood, liver, or kidney; and if active derivatives were stored, we should find that animals which had been plentifully supplied with vitamin A acid over long periods could withstand removal of this supplementation longer than they do. Our experiments show that such animals stop growing within a few days of deprivation, and develop severe symptoms of vitamin A deficiency within 1–2 weeks (cf. Fig. 5).

Vitamin A acid, however effective immediately, does not "buffer" the animal against deprivation. Having been formed irreversibly, it is apparently rapidly degraded; and it or its derivatives can exercise therefore only a transient function.[19] Ordinarily the rat must rely for vitamin A acid on the continuous oxidation of its stores of vitamin A. It is as the storable precursor of vitamin A acid that vitamin A probably fulfills its most important function.

This relationship may explain our observation that vitamin A is depleted from the liver at the same rate, whether or not the rat is supplied with vitamin A acid, as though this depletion proceeded independently of demand by the tissues. Such a condition might result automatically from the irreversible oxidation of vitamin A to the acid. A given amount—in young rats 2–2.5 μgm per day—may be oxidized to the acid in the liver or elsewhere, whether needed or not. Alternatively it is possible that the rat uses vitamin A preferentially as long as it remains available, perhaps because better equipped to transport it among the tissues, or for other reasons.

The animals maintained on vitamin A acid exhibit a sequence of changes, that seem to remain wholly restricted to the visual cells for as long as our experiments have continued: first, the decline in rhodopsin concentration, accounting completely for the rise of visual threshold; then the loss of opsin, and attendant disintegration of the outer segments of the rods; by this time also the retraction of the inner segments and irreversible decrease in number of the visual cells. Finally only a single, incomplete row of visual cell nuclei remains, devoid of inner segments. The animal is now blind, and apparently cannot recover its vision.

The retina in this final state (cf. Fig. 13) resembles closely that described in so-called "rodless" mice, a genetic condition associated probably with several distinct recessive mutations.[20, 21] In one such mutant strain (C3H), the retina develops normally until the mouse is 10 days old, outer and inner segments of the visual cells by then being clearly distinguishable. Thereafter, visual cells begin to die,

and by the age of about 25 days the retina has reached a state almost identical with that shown in Figure 13. A single row of stripped visual cell nuclei remains, the retina and pigment epithelium appearing otherwise normal, as they continue to do in the adult.[21]

The same histological pattern appears also in Johnson's description of a rat which, having been severely vitamin A-deficient, was allowed to "recover" through 12 weeks of vitamin A supplementation (cf. Johnson's Fig. 2D[22]). Again a single row of visual cell nuclei remains, devoid of inner or outer segments, in an otherwise relatively normal retina.

This condition also is characteristic of the human disease, *retinitis pigmentosa*.[23] Here again, though not invariably, the visual cell layer may come to contain finally a single row of nuclei, lacking inner and outer segments, though the bipolar and ganglion cell layers appear to remain intact. It has been suggested that this disease may involve some form of localized vitamin A deficiency.[23] One difficulty with this notion, however, is that in true vitamin A deficiency, degenerative changes are not confined to the visual cells, but may involve eventually all the retinal layers.[22, 24] One can, however, as the present experiments show, induce retinal changes closely comparable with those of *retinitis pigmentosa* by keeping animals on a vitamin A-deficient diet supplemented with vitamin A acid. The invasion of the retina by pigment is a secondary aspect of this disease, not to be looked for of course in our albino animals.

A characteristic feature of human *retinitis pigmentosa* is that the rods deteriorate before the cones.[23, 25] We have reason to anticipate the same sequence in vitamin A deficiency, whether or not supplemented with vitamin A acid. Cone visual pigments are synthesized very much more rapidly than rod pigments,[11] so that when vitamin A is in short supply, the cones may capture all they require, while the rods go hungry. (Cf. ref. 25). We have not yet succeeded in testing this point adequately, a difficult business in any case in the rat, in which cone vision seems to play only a minor role.

We have suggested mechanisms for the rise of visual threshold and the loss of rod outer segments in animals kept on the vitamin A-deficient diet supplemented with vitamin A acid. Why, however, when their other tissues remain intact, do such animals lose entire visual cells? This process seems to bear some relationship to certain types of traumatic degeneration known to occur in the retina and brain.[26] So, for example, when mammalian optic nerve fibers are cut, not only do their cells of origin, the retinal ganglion cells, degenerate ("retrograde degeneration"), but so also do cells of the lateral geniculate nucleus with which they make synaptic connection ("trans-synaptic degeneration"). Injury commonly has the effect of causing the degeneration of cells in the central nervous system; and it has been suggested that the subsequent degeneration of other cells with which they communicate may be caused by lack of excitation. Either explanation might be applied to the visual cells. They may go because the disintegration of their outer segments constitutes an "injury", even though this does not seem to deprive them of any vital part. On the other hand, the loss of their outer segments does deprive these cells of stimulation, and this may be a more powerful factor in their decay.

The loss of visual cells, so far as our experiments have gone, ends with the tissue

reduced to a single layer, in which the cells have lost all the special structures that marked them formerly as rods and cones; a condition shared, as already noted, with genetically "rodless" mice, and certain instances of human *retinitis pigmentosa*. At this point what remains of the layer of visual cells has the appearance of a simple, cuboidal epithelium (cf. Fig. 13). It faces another such simple epithelium, the pigment epithelium. These two tissues have the same embryonic origin, arising as adjoining patches lining the inner surface of the primary optic vesicle, composed of such potentially ciliated ependymal cells as ordinarily line the ventricles of the brain. Having come far apart in their subsequent development, they have been brought by the regression of the visual cells to resemble each other again to a remarkable degree.

Summary.—The general tissue functions of vitamin A that support growth and maintenance in the rat are served also by vitamin A acid; but since this substance is not reduced, it forms neither the alcohol, the form in which vitamin A is stored, nor the aldehyde (retinene) needed for the synthesis of visual pigments. For this reason, rats maintained on vitamin A acid, though growing normally and otherwise in good condition, become extremely night-blind, and eventually blind. The failure to form visual pigments also has specific anatomical consequences: the outer segments of the visual cells deteriorate, followed by the loss of almost all the cells themselves, in an otherwise normal retina. These anatomical changes resemble those observed in certain hereditary forms of blindness and in human *retinitis pigmentosa*.

* This investigation has been supported in part by grants from the Rockefeller Foundation, the Office of Naval Research, and the U.S. Public Health Service. Some of the measurements have been reported earlier (Dowling, J. E., and G. Wald, *Fed. Proc.*, **18**, 217 (1959); *Symposium on Vitamin Metabolism, Proc. IV Internat. Congress Biochemistry, Vienna*, **1958** (London: Pergamon Press, 1959). Part of this work was done while J. E. D. held a Post-Sophomore Fellowship of the Public Health Service, on leave from Harvard Medical School (1959–1960).

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We should also like to thank Dr. Aaron Ladman of the Harvard Medical School for preparing the retinal sections shown in Fig. 2.

[1] Wald, G., *Amer. J. Ophth.*, **40**, 18 (1955); *Exp. Cell Research*, Suppl. **5**, 389 (1958); Wald, G. and P. K. Brown, *Nature*, **177**, 174 (1956); Wald, G., P. K. Brown, and P. H. Smith, *J. Gen. Physiol.*, **38**, 623 (1954–1955).

[2] Dowling, J. E., and G. Wald, these *Proceedings*, **44**, 648 (1958); *Ann. N. Y. Acad. Sci.*, **74**, 256 (1958).

[3] Hubbard, R., *J. Gen. Physiol.*, **42**, 259 (1958–1959).

[4] Radding, C. M., and G. Wald, *J. Gen. Physiol.*, **39**, 923 (1955–1956).

[5] Arens, J. F., and D. A. Van Dorp, *Nature*, **57**, 190 (1946); Van Dorp, D. A., and J. F. Arens, *Rec. Trav. chim. Pays-Bas*, **65**, 338 (1946).

[6] Van Dorp, D. A., and J. F. Arens, *Nature*, **158**, 60 (1946).

[7] Sharman, I. M., *Brit. J. Nutr.*, **3**, viii (1949).

[8] Arens, J. F. and D. A. Van Dorp, *Nature*, **158**, 622 (1946).

[9] Moore, T., in *Symposium on Nutrition*, ed. R. M. Herriott, Johns Hopkins Press, (Baltimore: 1953), p. 28. Dr. Moore's words were: "Perhaps I may be allowed to suggest to Dr. Wald that it would be very interesting to examine the effect of vitamin A acid, if any, in curing defective dark adaptation in animals deficient in vitamin A." He had already explained that he thought it probably would have no effect. See also the discussion of these relationships in Moore's book, *Vitamin A* (Amsterdam: Elsevier Publ. Co., 1957), pp. 282–283.

[10] Dowling, J. E., unpublished experiments, cited in Dowling, J. E., and G. Wald, *Symposium in Vitamin Metabolism, Proc. IV Internat. Congress, Biochemistry, Vienna, 1958* (London: Pergamon Press, 1959); also in Wald, G., in *The Squibb Centennial Lectures* (New York: G. P. Putnam's Sons, 1959), p. 49.

[11] The first such measurements were performed in normal and vitamin A-deficient rats by K. Tansley, *J. Physiol.*, **71**, 442 (1931). She assumed, as the simplest relation, that the sensitivity (1/threshold) is proportional to the concentration of rhodopsin. We find the *logarithm* of the sensitivity to exhibit this proportionality. Compare also ophthalmoscopic measurements of the bleaching and regeneration of rhodopsin in the rat retina during light and dark adaptation, by Lewis, D. M., *J. Physiol.*, **136**; 615, 624 (1957).

[12] Wald, G., P. K. Brown, and P. H. Smith, *J. Gen. Physiol.*, **38**, 623 (1954–1955).

[13] Dowling, J. E., and I. R. Gibbons, in *Symposium on Eye Structure, Proc. Internat. Congress Anatomists, New York, April 11–16, 1960* (to be published by Academic Press, New York).

[14] Hecht, S., and J. Mandelbaum, *Science*, **88**, 219 (1938); *J. Am. Med. Assoc.*, **112**, 1910 (1939); *Amer. J. Physiol.*, **130**, 651 (1940); McDonald, R., and F. H. Adler, *Arch. Ophth.*, **24**, 447 (1940).

[15] de Robertis, E., *J. Biophys. Biochem. Cytol.*, **2**, 319 (1956); **2** (Suppl.), 209 (1956).

[16] Glover, J., T. W. Goodwin, and R. A. Morton, *Biochem. J.*, **41**, 97 (1947); Krinsky, N. I., D. G. Cornwell, and J. L. Oncley, *Arch. Biochem. Biophys.*, **73**, 233 (1958).

[17] Bliss, A. F., *Arch. Biochem. Biophys.*, **31**, 197 (1951).

[18] Wald, G., and R. Hubbard, these PROCEEDINGS, **36**, 92 (1950). The enzyme, retinene reductase, mentioned in this paper appears to be identical with liver alcohol dehydrogenase [Bliss, A. F., *Biol. Bull.*, **97**, 221 (1949)].

[19] The degradation of vitamin A in the rat has recently been examined (Wolf, G., S. G. Kahn, and B. C. Johnson, *J. Amer. Chem. Soc.*, **79**, 1208 (1957). Large amounts of vitamin A (3–4 mgm) labeled with penultimate C^{14}, were injected intraperitoneally. Surprisingly large fractions were excreted in the form of water-soluble, apparently aliphatic, compounds in the urine.

[20] Keeler, C. E., these PROCEEDINGS, **12**, 255 (1926); *J. Exp. Zool.*, **46**, 355 (1937).

[21] Noell, W., *Arch. Ophthal.*, **60**, 725 (1958); *Ann. N. Y. Acad. Sci.*, **74**, 355 (1958).

[22] Johnson, M., *Arch. Ophthal.*, **29**, 793 (1943).

[23] Cogan, D. G., *Trans. Amer. Acad. Ophthal. and Otolar.*, July-August, 1950, p. 629

[24] Tansley, K., *Proc. Roy. Soc. London*, **114**, 79 (1933); Johnson, M. L., *J. Exp. Zool.*, **81**, 67 (1939).

[25] Zeavin, B. H., and G. Wald, *Amer. J. Ophthal.*, **42**, 253 (1956).

[26] Cf. discussion of axonal (retrograde) and transneuronal degeneration in Polyak, S. L., *The Retina* (University of Chicago Press, 1941), pp. 68–72.

EVIDENCE FOR A VICINAL DITHIOL IN OXIDATIVE PHOSPHORYLATION

BY ARVAN FLUHARTY AND D. R. SANADI

GERONTOLOGY BRANCH, NATIONAL HEART INSTITUTE, NATIONAL INSTITUTES OF HEALTH, AND THE BALTIMORE CITY HOSPITALS

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The phosphorylation associated with electron transport in the respiratory chain can be uncoupled by physical damage to the structure of mitochondria or by a variety of special compounds. Several investigators have studied the mechanism of action of these compounds, 2,4-dinitrophenol (DNP) in particular, in an attempt to elucidate the coupling process. One more compound was added to the

already large list when Jacobs *et al.*[1] observed that Cd^{++} was effective at very low levels in uncoupling phosphorylation with no significant effect on oxidation. The uncoupling was reversed readily by compounds with vicinal dithiols but not by monothiols which suggested the possible involvement of vicinal dithiols in oxidative phosphorylation. Such a dithiol could serve as a system capable of trapping the energy of electron transport as in the case of the oxidative decarboxylation of α-keto acids.[2-4]

The intervention of a vicinal dithiol compound in biological reactions was postulated by Peters[5] in the course of studies on the action of arsenicals on the pyruvic oxidase system. The demonstration of 6,8-thioctic acid (α-lipoic acid), a compound which on reduction yields such an arrangement of -SH groups, as an integral part of the α-keto acid oxidase systems[2, 3] established the value of arsenite and arsenicals as reagents for detecting functional vicinal dithiols in enzymatic reactions. Jacoby[6] has studied aldehyde dehydrogenases from a number of sources and, on the basis of arsenite inhibition and reversal by 2,3-dimercaptopropanol (BAL), has postulated the involvement of closely juxtaposed sulfhydryl groups in this reaction. The dihydrothioctyl dehydrogenase derived from α-ketoglutaric dehydrogenase also meets the criteria for an enzyme with vicinal dithiols.[8] The dithiol-disulfide in this case plays an oxidation-reduction role functional in the interaction with thioctyl derivatives but not in the reduction of artificial electron acceptors (diaphorase reaction).

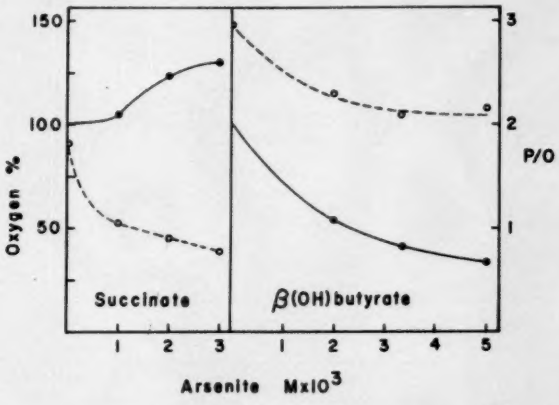


FIG. 1.—*Effect of arsenite on oxidation and coupled phosphorylation.* The rat liver mitochondrial preparation was preincubated with sodium arsenite at 0° in 1 ml of 0.3 *M* sucrose for 10 min. This suspension was then added to the reaction system in Warburg flasks to make a final volume of 3 ml. The experimental conditions were essentially as described previously[1] except that fluoride was not used. The final concentration of arsenite in 3 ml is shown in the figure. Key: •——•, Oxygen consumption. O-----O, Ratio of moles phosphate esterified to equivalents of oxygen consumed (P/O).

On the basis of available data it is clear that arsenite binds vicinal dithiols far more strongly than it binds monothiols. Inhibition of a vicinal dithiol system is apparent at much lower concentrations of arsenite (of the order of 10^{-4} *M*) than the levels necessary to show significant inhibition with a monothiol enzyme (10^{-3} *M* or greater). Due to this greater affinity, inhibition of a dithiol enzyme by arsenite often occurs even in the presence of a large excess of cysteine or mercaptoethanol.[6, 7] Also, reversal of the inhibition is observed more readily with other dithiols than with monothiols.[6-8]

Previous studies on the effect of arsenite on oxidative phosphorylation have been inconclusive. Lehninger,[9] using a particulate fraction from rat liver, found that $9 \times 10^{-3}M$ sodium arsenite completely inhibited the incorporation of P^{32}-labeled phosphate into ATP but had no effect on the oxidation of β-hydroxybutyrate.

Hunter and Ford,[10] on the other hand, observed inhibition of oxidation and concluded that arsenite had a non-specific effect on mitochondrial structure. Swanson[11] also found that the P^{32}-phosphate exchange with ATP which occurred independent of oxidation was inhibited and ATPase activity was stimulated by 10^{-3} to 10^{-2} M arsenite; effects on oxidation were not determined. Crane and Lipmann[12] found that 1×10^{-4} M arsenite had no effect on oxidative phosphorylation with succinate.

Results.—The effect of arsenite on the oxidation of succinate and β-hydroxybutyrate and on phosphate esterification coupled to the oxidation is shown in Figure 1. The rat liver mitochondria were preincubated in 0.3 M sucrose at 0° for 5–10 min with arsenite which was previously adjusted to pH 7.2. One ml of the suspension was added to Warburg flasks and the assay was carried out in 3.0 ml as described previously.[1] The figures and tables show the final concentrations of the components in the reaction mixture. The oxidation of succinate was stimulated at 1 to 3×10^{-3} M arsenite but the associated phosphate esterification was partially uncoupled. The P/O ratio generally decreased from a region around two to a region around one. The decrease in the value never exceeded one unit. Over a similar range of arsenite concentration, β-hydroxybutyrate oxidation was inhibited usually 50 to 80 per cent, but the P/O ratio declined only slightly. The oxidation of glutamate and also that of citrate and malate were strongly inhibited by arsenite, 50 per cent inhibition being obtained at 4.0×10^{-6} M, 3.0×10^{-5} M, and 4.5×10^{-5} M respectively. Although roughly parallel uncoupling was observed, it was difficult to evaluate the significance of the result in view of the resultant low oxygen uptake.

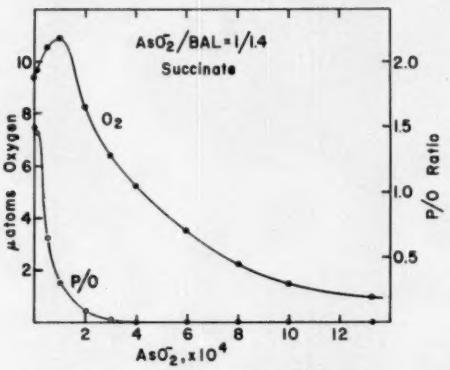


FIG. 2—*Effect of arsenite-BAL combination on the oxidation of succinate and accompanying phosphorylation.* The arsenite and BAL were preincubated with the mitochondrial preparation in 1 ml. of 0.3 M sucrose with the assay being carried out as in Fig. 1. In all cases BAL was present at 1.4 times the concentration of arsenite. Key: •——•, Oxygen consumption. O------O, P/O.

When attempts were made to reverse the uncoupling effects in the succinate system by the addition of 2,3-dimercaptopropanol (BAL) to the arsenite-treated mitochondria, inhibition of oxygen uptake was obtained although BAL alone caused no change in the oxidation. This surprising effect of the BAL-arsenite combination is shown in Figure 2. A significant increase in the oxidation rate accompanied by complete uncoupling of phosphorylation was observed at concentrations around 10^{-4} M. As the concentration of BAL-arsenite was increased, the oxidation was also inhibited. In these experiments the order of addition of BAL and arsenite to the preincubation mixture had no influence; similar results were obtained whether arsenite or BAL was added first to mitochondria or BAL and arsenite were premixed immediately before the addition to mitochondria.

The pronounced inhibitory effect of low levels of arsenite (1 to 2×10^{-5} M) on glutamate oxidation was completely reversed by an equivalent concentration

of BAL; in fact, a stimulation of oxidation was observed (BAL alone gave similar stimulation) with efficient phosphorylation (data not shown). An increase in the BAL-arsenite combination to 1 to 3 $\times$ 10^{-4} M produced strong inhibition of oxidation of glutamate as well as of β-hydroxybutyrate (Table 1, line 2). Addition of DPN restored the oxidation partially (line 4) and revealed the fact that phosphorylation was uncoupled just as in succinate oxidation. The data with citrate and malate as substrates was again consistent with the above results. Arsenite-BAL combination (1 $\times$ 10^{-4} M) inhibited oxygen uptake which was partially restored by DPN, but not by TPN, and almost completely uncoupled phosphorylation (data not shown). The uncoupling occurred at roughly the same level with all these substrates. The DPN stimulated oxidations were inhibited by antimycin A.

Reversal by excess BAL: If mitochondria were inhibited by exposure to arsenite and equivalent BAL, and then over a 10-fold excess of BAL was added, the activity of the system was largely restored (Fig. 3 and Table 1). With β-hydroxybutyrate and glutamate as substrates, the oxidation also was fully restored even without any DPN added. The concentration of arsenite was selected to provide a reasonable demonstration of reversal although the initial uncoupling was not complete.

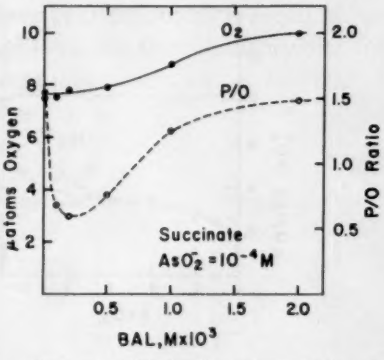


FIG. 3.—*Effect of various levels of BAL on oxidation and phosphorylation in the presence of a constant level of arsenite.* The mitochondria were incubated as in Fig. 1 with arsenite for 5 min at 0°. Then BAL was added as indicated. The final concentration of arsenite was 1 $\times$ $10^{-4}M$. Key: •——•, Oxygen uptake. O----O, P/O.

The effects of BAL-arsenite combination on oxidation and on phosphorylation could be duplicated if the BAL were replaced by other dithiol compounds (e.g., sodium dihydrothioctate and dihydrothioctamide) but not by monothiol compounds (e.g., cysteine, glutathione, mercaptoethanol, thioglycollate at equivalent levels or in 10-fold excess).

Reversal of the effect of BAL-arsenite could be obtained by monothiols (cysteine, mercaptoethanol) also, but only at levels as high as 7 $\times$ 10^{-2} M. Reversal by dithiol compounds other than BAL was not attempted.

ATPase: It has been established that freshly prepared mitochondria have little ATPase activity with or without Mg^{++} and that DNP exposes an active ATPase.[13, 14] In agreement with the observation of Swanson,[11] mitochondria showed significant ATPase activity in the presence of 1 $\times$ 10^{-3} M arsenite (Fig. 4). The additional activation by Mg^{++} is indicative of structural changes in mitochondria in the presence of these levels of arsenite. Arsenite, however, had no effect on the DNP-activated ATPase. A considerably lower concentration of arsenite (1 $\times$ 10^{-4} M), in combination with a similar level of BAL, produced a striking activation of ATPase activity similar to that observed with DNP. Increase in the BAL-arsenite combination produced parallel inhibitions of the ATPase in the two systems. Addition of excess BAL decreased the ATPase activity of the BAL-arsenite treated mitochondria both alone and in the presence of Mg^{++} (Fig. 4). The data indicate a trend towards reversal of the effects of BAL-arsenite

and restoration of the original mitochondrial activities. DPN has no such effect on the ATPase under any set of conditions studied so far.

The effects on ATP-P^{32} exchange were similar; 5×19^{-5} *M* arsenite gave 51 per cent inhibition and 5×10^{-5} *M* BAL gave 26 per cent activation. The two together gave 93 per cent inhibition which was comparable to the 89 per cent obtained with 10^{-4} *M* DNP.

Preliminary studies have shown that particulate fragments prepared by osmotic lysis of mitochondria show a similar requirement for BAL besides arsenite for uncoupling of phosphorylation with succinate as substrate.

Discussion.—The effect of arsenite on oxidation and coupled phosphorylation with succinate and β-hydroxybutyrate as substrates become significant only at high levels. At these concentrations, thioarsenite formation with monothiols and structural changes in mitochondria are possible.[10] The sensitivity of glutamate, citrate, and malate oxidation to arsenite is of considerable interest and warrants a more detailed examination.

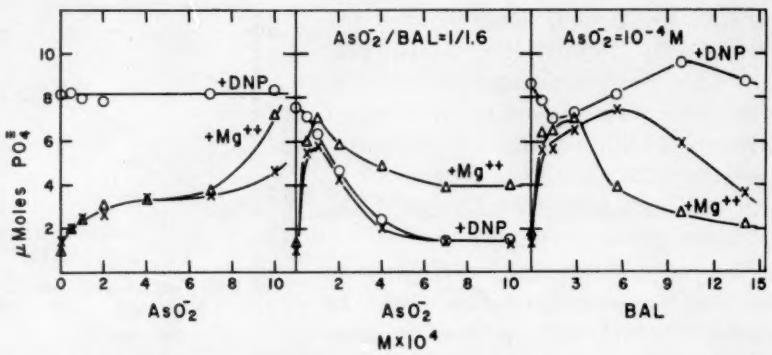


FIG. 4.—*Effect of arsenite and arsenite-BAL combination on ATPase activity.* The mitochondrial preparation was preincubated as in Figure 1 and 2 with arsenite and arsenite-BAL. ATPase activity was determined by adding 0.33 ml. of the suspension to the remaining reaction components[13] to give a final volume of 1 ml. BAL and arsenite concentrations refer to the level in the final assay mixture. Key: X——X Endogenous activity. ○——○ 3×10^{-5} *M* DNP was also present in the final assay mixture. Δ——Δ 5×10^{-3} *M* $MgCl_2$ was added to assay mixture.

The presence of a dithiol compound together with low concentrations of arsenite produces striking uncoupling of phosphorylation with slight stimulation of oxidation with succinate. In the case of pyridine nucleotide linked substrates, the oxidation is strongly inhibited but addition of DPN largely restores the oxidation, and the uncoupling effect becomes apparent. Since an excess of BAL also restores the oxidation, it would appear that DPN is still present within the mitochondria but may not be available for the dehydrogenases.

In order to decide between two alternative explanations for the uncoupling effect of the BAL-arsenite combination, attempts were made to reverse the phenomenon by the addition of excess BAL. If the cyclic thioarsenite of BAL *per se* were the uncoupling agent, addition of excess BAL would not be expected to have any effect. On the other hand, if the combination of arsenite with BAL was merely a device to carry the arsenite to the active center, which may be another

dithiol, transfer of the arsenite to the enzyme dithiol may occur to an extent determined by the relative affinities of the two dithiols. Accordingly BAL would be a carrier of arsenite either by virtue of the lower charge on the complex per unit mass or of its increased lipid solubility. If the second hypothesis were true, addition of excess BAL would be expected to remove some of the arsenite bound to the enzyme by mass action effect. The data clearly show a reversal of uncoupling of phosphorylation by a tenfold excess of BAL and are consistent with the second explanation (Fig. 3 and Table 1).

The effects on ATPase activity of mitochondria are a perfect complement of the above effects on phosphorylation. Arsenite by itself stimulates the ATPase with or without added Mg^{++} but only at high levels (Fig. 4). It has no effect on the DNP-activated ATPase. In combination with BAL, low levels of arsenite (1×10^{-4} *M*) markedly enhance ATPase activity almost to the level of DNP-activated ATPase. The ATPase activity, both with and without Mg^{++}, is lowered when a tenfold excess of BAL is added to the system which is indicative of reversal of the effects of BAL-arsenite. The relative affinities of BAL and the inhibitory site for arsenite must be of such magnitude that a clearer demonstration of reversal of

TABLE 1

REVERSAL OF UNCOUPLING BY EXCESS BAL

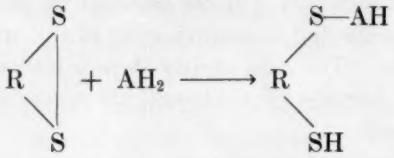
	Additions	β(OH) Butyrate		Glutamate	
		Oxygen atoms	P/O	Oxygen atoms	P/
1	None	5.8	2.1	6.2	2.4
2	BAL-AsO_2	2.2	0.5	0.8	0
3	BAL-AsO_2, Xs BAL	6.2	1.4	4.8	1.4
4	BAL-AsO_2, DPN	6.0	0.2	3.0	0
5	BAL-AsO_2, Xs BAL, DPN	7.4	1.5	5.4	1.5
6	None	...	...	6.1	2.9
7	AsO_2^-	...	...	1.7	3.0
8	DPN	...	...	5.5	2.8
9	AsO_2^-, DPN	...	...	1.7	2.8

The experiment was carried out as in Fig. 3 with BAL-arsenite (1.3×10^{-3} *M* and 1.0×10^{-3} *M* respectively, combination) excess BAL (1.0×10^{-2} *M*); DPN (1.0×10^{-3} *M*) and arsenite in lines 7 and 9 (1.0×10^{-5} *M*).

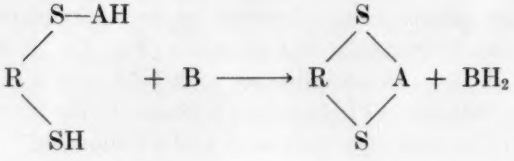
uncoupling and ATPase appearance is difficult. DPN had no effect on the ATPase activity which is consistent with its inability to reverse the uncoupling effect.

Under our experimental conditions the optimal effect is obtained at approximately 1×10^{-4} *M* arsenite and roughly equivalent BAL. When the concentration of the complex (at the same ratio of BAL to arsenite) is increased, an inhibition of both oxidation (Fig. 2) and ATPase activity (Fig. 4) is observed. This is in contrast to the effect of increasing the concentration of DNP on ATPase.[13,15] Also the BAL-arsenite complex, at increasing concentrations, produces a remarkably parallel inhibition of the DNP-activated ATPase (Fig. 4). These results are significant from the standpoint of mechanism. It would appear that the inhibition at high levels of BAL-arsenite is due to the blocking of another site. Since DNP does not reverse the inhibition of oxidation produced by 10^{-3} M BAL-arsenite, either this secondary site is identical with the point of interaction of DNP with the oxidative phosphorylation system, or two sites (one in the respiratory chain and the second concerned with the energy transfer reactions) are simultaneously inhibited.

A mechanism of oxidative phosphorylation that would explain the observations is shown in equations (1) to (5). It has some similarity to the proposed primary energy-trapping reaction in the oxidation of α-keto acids.[2-4]



(1)



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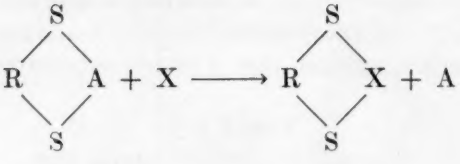

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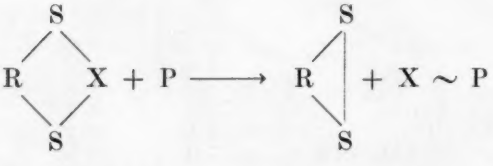
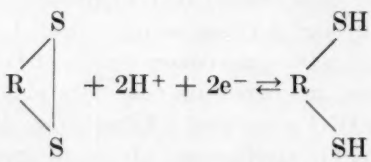


(4)

$$Y \sim P + ADP \longrightarrow ATP + X \tag{5}$$

It assumes that the reduced carrier (AH_2) can react with the disulfide (Reaction 1) and exist as a charge transfer complex analogous to the complexes noted by Isenberg and Szent-Gyorgyi.[16] In a phosphorylating system this complex is the substrate for oxidation by the next electron carrier (Reaction 2) resulting in the capture of oxidative energy. In preparations of electron transport particles devoid of phosphorylative coupling, AH_2 is directly oxidized (Reaction 6) without the intervention of the disulfide. The inhibition by arsenite-BAL would require

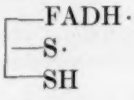
$$AH_2 + B \longrightarrow A + BH_2 \tag{6}$$



(7)

that the disulfide is in equilibrium with the dithiol (equation (7)). In these equations, R could be a small molecular weight compound or a protein or even two protein chains. The only requirement is that the two —SH groups should be in juxtaposition. If the —SH groups are on a small molecule similar to thioctic acid, the strain energy of the ring system[17] would be available for changing the equilibrium of Reaction 1 in the desired direction.

Evidence for an interaction of the type postulated in Reaction 1 between a disulfide group and a reduced coenzyme has been observed in the case of dihydrothioctyl dehydrogenase which has a flavinadeninedinucleotide (FAD) and a reducible disulfide group on the same protein.[8] On partial reduction of the enzyme with DPNH an absorption maximum indicative of a flavin semiquinone is formed near 520 mμ. The disappearance of this peak on the addition of arsenite or Cd^{++} suggests that the semiquinone is stabilized by resonance interaction between the reduced flavin and disulfide which may be represented as



The inhibition of the exchange of labeled phosphate with ATP indicates the participation of the disulfide in the reaction where inorganic phosphate is activated. It is of interest to note that the soluble exchange enzyme of Plaut[18, 19] is not inhibited by arsenite. The effect of BAL-arsenite on the terminal ADP-ATP exchange[20] has not been examined.

It may be significant that all of the enzyme systems which have functional dithiols defined by high sensitivity to arsenite and reversal by added dithiol compounds are concerned with oxidative reactions.[2, 5–8, 20–22] Since some of these are coupled with energy conservation, it would be of interest to examine whether the other reactions could also be linked to phosphorylation.

The authors wish to thank Miss Jo Ann Swartz for her excellent technical assistance.

Note added in proof: From theoretical considerations Dr. Henry Mahler[23] has speculated on a possible interaction between a disulfide and an oxidized electron carrier as a preliminary step in oxidative phosphorylation.

[1] Jacobs, E. E., M. Jacob, D. R. Sanadi, and L. B. Bradley, *J. Biol. Chem.*, **223**, 147 (1956).

[2] Gunsalus, I. C., in *The Mechanism of Enzyme Action*, ed. W. D. McElroy and B. Glass (Baltimore: Johns Hopkins, 1954), p. 545.

[3] Reed, L. J., *Physiol. Rev.*, **33**, 544 (1953).

[4] Calvin, M., and P. Massini, *Experientia*, **8**, 445 (1952).

[5] Peters, R. A., *Symp. Soc. Exptl. Biol.*, **3**, 36 (1949).

[6] Jacoby, W. B., *J. Biol. Chem.*, **232**, 89 (1958).

[7] Sanadi, D. R., M. Langley, and F. White, *J. Biol. Chem.*, **234**, 183 (1959).

[8] Searls, R. L., and D. R. Sanadi, *Biochem. Biophys. Res. Comm.*, **2**, 189 (1960).

[9] Lehninger, A. L., *J. Biol. Chem.*, **178**, 625 (1949).

[10] Hunter, F. E., Jr., and L. Ford, *J. Biol. Chem.*, **216**, 357 (1955).

[11] Swanson, M., *Biochem. Biophys. Acta*, **20**, 85 (1956).

[12] Crane, R. K., and F. Lipmann, *J. Biol. Chem.*, **201**, 235 (1953).

[13] Lardy, H. A., and H. Wellman, *J. Biol. Chem.*, **201**, 357 (1953).

[14] Hunter, F. E., Jr., in *Phosphorus Metabolism*, ed. W. B. McElroy and B. Glass (Baltimore: Johns Hopkins, 1951), Vol. 1, p. 297.

[15] Lardy, H. A., and H. Wellman, *J. Biol. Chem.*, **195**, 215 (1952).

[16] Isenberg, I., and A. Szent-Gyorgi, these PROCEEDINGS, **44**, 857 (1958); **45**, 1229 (1959).

[17] Barltrop, J. A., P. M. Hayes, and M. Calvin, *J. Amer. Chem. Soc.*, **76**, 4348 (1954).

[18] Plaut, G. W. E., *Arch. Biochem. Biophys.*, **69**, 320 (1957).

[19] Chiga, W., and G. W. E. Plaut, *J. Biol. Chem.*, **234**, 3059 (1959).

[20] Wadkins, C. L., and A. L. Lehninger, *J. Biol. Chem.*, **233**, 1589 (1958).

[21] Mahler, H. R., B. Mackler, D. E. Green, and R. M. Bock, *J. Biol. Chem.*, **210**, 465 (1954).
[22] Mackler, B., H. R. Mahler, and D. E. Green, *J. Biol. Chem.*, **210**, 149 (1954).
[23] Mahler, G. R. (private communication).

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*THEORETICAL ANALYSIS OF TRANSPORT PROCESSES IN LIVING SYSTEMS**

BY OLEG JARDETZKY† AND FRED M. SNELL

DEPARTMENTS OF PHARMACOLOGY AND BIOLOGICAL CHEMISTRY, HARVARD MEDICAL SCHOOL, AND DEPARTMENT OF BIOPHYSICS, UNIVERSITY OF BUFFALO SCHOOL OF MEDICINE

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The ability of living cells to create and maintain differences in their composition from that of their environment must in many cases depend on the existence of specific mechanisms for the selective transport of certain molecular species as opposed to others. Neither differences in permeability to various species migrating at random nor alterations in structure of the intracellular medium are sufficient to explain all the observations made to date. Such specific processes peculiar to living systems are intuitively understood under the term *active transport.*

In recent years appreciable effort has been made to so refine the concept of active transport as to be able to ascertain experimentally (1) whether in a given system a given molecular component has been transported by a specific mechanism and (2) if so, what fraction of the total amount transported can be accounted for in terms of transport by this mechanism.[1–4] Although the proposed definitions of active transport have served well to guide experimentation, none of them are satisfactory in themselves inasmuch as they are either not sufficiently inclusive or sufficiently rigorous. The various formulations and the specific ambiguities associated with them have been discussed in several earlier papers.[5–10]

It is our purpose in this paper to present the concepts essential to a systematic description of transport processes in living systems and to show that the types of transport phenomena generally considered to be peculiar to such systems can be formulated in rigorous terms. In approaching the problem of describing any phenomena occurring in living systems, one must bear in mind the inherent complexity of such systems. This complexity will unavoidably be reflected in their description in mathematical terms. Thus the cell contains somewhere between 10^3 and 10^5 distinguishable molecular species, and probably as many transport or transformation processes, all occurring simultaneously. Even though the physical laws governing each individual process are as simple as they would be, were the process to be observed in isolation, their interdependence must be explicitly taken into account. For this purpose we have utilized in the following treatment the notation current in thermodynamics of irreversible processes. It should be emphasized, however, that the adoption of this convenient notation does not constitute an application to this problem of the thermodynamic theory of irreversible processes as such. In particular, the validity of the treatment does not depend either on an assumption of a linear relationship between fluxes and forces or on the Onsager reciprocal relations.

Mathematical Theory.—We choose for analysis a representative living system in contact with and open to its environment. All processes occurring within it and across its boundaries may be completely described by sets of equations of the form:[11, 12]

$$\vec{J}_i = \sum_j^n \lambda_{ij}\vec{X}_j \qquad (i = 1, 2, \ldots n) \tag{1}$$

$$J_\alpha = \sum_\epsilon^\rho \lambda_{\alpha\epsilon}A_\epsilon \qquad (\alpha = 1, 2, \ldots \rho) \tag{2}$$

where $\vec{J}_i$ is the vectorial flux of quantity i, X_j is the jth thermodynamic force and λ_{ij} is the coefficient relating the two. In general, λ_{ij} is a function of $\vec{X}_j$. J_α is the rate of the α^{th} reaction, defined as

$$J_\alpha \nu_{\alpha i} = \frac{1}{V}\frac{d_\alpha n_i}{dt} \tag{3}$$

where $\nu_{\alpha i}$ is the stoichiometric coefficient for the i^{th} constituent in the α^{th} reaction, V the volume of the region in which the reaction is occurring, and $d_\alpha n_i/dt$ is the rate of change of the number of moles of constituent i due to the α^{th} reaction. A_ϵ is the thermodynamic force, driving the ϵ reaction, the so-called affinity, and $\lambda_{\alpha\epsilon}$ is a coefficient which in general is a function of A_ϵ. The two separate sets of equations, (1) and (2), are used to describe transport and transformation phenomena, respectively, because it is necessary to distinguish the different tensorial character of the thermodynamic forces involved, inasmuch as Curie's theorem states that it is impossible for a force of a given tensorial order to be associated with a flow of a higher tensorial order.[13]

It follows, therefore, that the one and only way to effect a transport process by the scalar forces of chemical reactions is to have a source and a sink set up by such reactions in distinct but contiguous regions of space. One may thus visualize the production of a constituent by a chemical reaction in one region of space and its destruction in another region, giving rise to a thermodynamic vectorial force and a concomitant flow.

Without loss of generality and for purposes of simplification, we shall consider in the following formulations two adjacent regions of space separated by a boundary and in which ρ and ρ' reactions respectively may occur. The contribution of a chemical reaction to the transport of any constituent i may be taken as the effect of a change in the difference of its chemical potential between the two regions resulting from this reaction.[14] We are thus interested in evaluating this contribution in terms of the reaction rate.

A given reaction, α, occurring in one of the specified regions will produce a change in the quantity of constituent i according to equation (3). The total change in the quantity of the constituent i is given by

$$\frac{dn_i}{dt} = \frac{d^P n_i}{dt} + \frac{d^T n_i}{dt} = V\sum_\alpha^\rho J_\alpha \nu_{\alpha i} - \vec{S}\cdot\vec{J}_i \qquad (i = 1, 2, \ldots n) \tag{4}$$

where the superscripts P and T denote the total contributions of reactions and trans-

port respectively, and $\vec{S}$ is the vector area defined as $\int_s \vec{r} \cdot d\vec{S}$ where $\vec{r}$ is the unit vector perpendicular to an element of the surface, $d\vec{S}$.

The relation between the change in chemical potential of i resulting from changes in the quantities of constituents, j, due to the α^{th} reaction is given by

$$\frac{d_\alpha \mu_i}{dt} = \sum_j^n g_{ij} \frac{d_\alpha n_j}{dt} \qquad \begin{Bmatrix} i = 1, 2, \ldots n \\ \alpha = 1, 2, \ldots \rho \end{Bmatrix} \tag{5}$$

where $g_{ij} = \left(\frac{d\mu_i}{dn_j}\right)_{nk}$.

If we now define for a given region

$$\eta_{\alpha i} = \int_0^\tau \frac{d_\alpha \mu_i}{dt}\, dt \qquad \begin{Bmatrix} i = 1, 2, \ldots n \\ \alpha = 1, 2, \ldots \rho \end{Bmatrix} \tag{6}$$

where τ denotes an arbitrary period of observation, then η_i, the total contribution of chemical reactions to the chemical potential of constituent i for the interval τ in a region is given by

$$\eta_i = \sum_\alpha^\rho \eta_{\alpha i} \qquad (i = 1, 2 \ldots n) \tag{7}$$

and may be evaluated in terms of the reaction velocities with the use of equations (3), (5), and (6). For the two-compartment system under consideration the vector forces in the flux equations (1) may thus be expressed as

$$\vec{X}_i = -(\mu_i' - \mu_i) = -(\xi_i' - \xi_i + \eta_i' - \eta_i) = -(\Delta\xi_i + \Delta\eta_i) \qquad (i = 1, 2 \ldots n) \tag{8}$$

where

$$\xi_i = \xi_{i0} + \int_0^\tau \frac{d^{\mathrm{T}}\mu_i}{dt}\, dt = \xi_{i0} + \int_0^\tau \sum_j^n g_{ij} \vec{S} \cdot \vec{J}_j dt \qquad (i = 1, 2 \ldots n). \tag{9}$$

It is evident that ξ_i is the sum of the initial chemical potential, ξ_{i0}, and the change in μ_i brought about by transport processes in the period τ. From equations (1) and (8) it follows that the free flux of constituent i is

$$\vec{J}_i = -\sum_j^n \lambda_{ij} \Delta\xi_j - \sum_j^n \lambda_{ij} \Delta\eta_j = \vec{J}_i^{\,T} + \vec{J}_i^{\,\mathrm{P}} \tag{10}$$

where the first term J_i^T is that resulting from forces to which there has been no contribution from chemical reactions and the second, J_i^{P} accounted for exclusively in terms of sources and sinks resulting from reactions.

In a system in which the molecular structure of constituent i can be altered by addition or removal of a chemical group or by a change in conformation, to yield constituents k, transported in this form, and reconstituted to form constituent i upon completion of transport, the total resulting flux of i will be

$$\vec{J}_{iT} = \vec{J}_i + \sum_{\substack{k \\ k(i}}^m \nu_{ik} \vec{J}_{ik} \qquad (k = 1, 2 \ldots m). \tag{11}$$

The symbol, $k(i$, implies that k is derived from i, and ν_{ik} is the number of moles of i yielding one mole of k.

In addition i may be either incorporated into or released from other constituents, designated b, which are not subject to directed translational movement. Thus we must distinguish between two types of reactions both of which by involving constituent i contribute to the forces involved in its transport but in fundamentally different ways. In the former, the transport is effected by the flux of constituents k derived from i, whereas in the latter it results from an alteration of the chemical potential difference of constituent i itself, by direct reaction. There remains then only the contribution of those reactions which do not directly involve constituent i. Accordingly, we may divide the contribution resulting from chemical reactions into these categories:

$$\Delta\eta_i = \sum_{\alpha}^{\nu} \Delta\eta_{\alpha i} = \sum_{\kappa}^{K}\Delta\eta_{\kappa i} + \sum_{\beta}^{B}\Delta\eta_{\beta i} + \sum_{\gamma}^{\Gamma}\Delta\eta_{\gamma i}$$
$$= \Delta\eta_i^{K} + \Delta\eta_i^{B} + \Delta\eta_i^{\Gamma} \qquad (i = 1,2 \ldots n) \tag{12}$$

where κ are reactions leading to the formation or decomposition of k derived from i for which $\vec{J}_k \neq 0$; β, the reactions leading to the formation or decomposition of b containing i, for which $\vec{J}_b \equiv 0$; and γ, the remaining reactions not directly involving i.

We are now in the position to express the total flux of constituent i in terms of the individual contributions of different types of processes. From equations (10) and (11) we have

$$\vec{J}_{iT} = -\sum_{j}^{n} \lambda_{ij}\Delta\xi_j - \sum_{j}^{n} \lambda_{ij}\Delta\eta_j - \sum_{\substack{jk \\ k(i}}^{n} \nu_{ik}\lambda_{kj}\Delta\xi_j - \sum_{\substack{jk \\ k(i}}^{n} \nu_{ik}\lambda_{kj}\Delta\eta_j \qquad (i = 1, 2 \ldots n) \tag{13a}$$

wherefrom we obtain by subdividing the second term on the right in accordance with equation (12)

$$\vec{J}_{iT} = -\sum_{j}^{n} \lambda_{ij}\Delta\xi_j - \sum_{\substack{jk \\ k(i}}^{n} \nu_{ik}\lambda_{kj}\Delta\xi_j - \lambda_{ii}(\Delta\eta_i^{K} + \Delta\eta_i^{B} + \Delta\eta_i^{\Gamma})$$
$$- \sum_{j}^{n} \lambda_{ij}\Delta\eta_j - \sum_{\substack{jk \\ k(i}}^{n} \nu_{ik}\lambda_{kj}\Delta\eta_j \qquad (i = 1, 2 \ldots n). \tag{13b}$$

On the basis of the origin of the thermodynamic forces involved, it is thus possible to separate the total flux of any component i into four distinct terms.

$$\vec{J}_{iP} = -\sum_{j}^{n} \lambda_{ij}\Delta\xi_j - \sum_{\substack{jk \\ k(i}}^{n} \nu_{ik}\lambda_{kj}\Delta\xi_j \tag{14a}$$

$$\vec{J}_{iA} = -\sum_{\substack{jk \\ k(i}}^{n} \nu_{ik}\lambda_{kj}\Delta\eta_j - \sum_{\substack{j \\ j\neq i}}^{n} \lambda_{ij}\Delta\eta_j \tag{14b}$$

$$\vec{J}_{iB} = \lambda_{ii}(\Delta\eta_i^{B} + \Delta\eta_i^{\Gamma}) \tag{14c}$$

$$\vec{J}_{iR} = -\lambda_{ii}\Delta\eta_i^{K} \tag{14d}$$

$$\vec{J}_{iT} = \vec{J}_{iP} + J_{iA} + J_{iB} + \vec{J}_{iR} \qquad (i = 1, 2 \ldots n). \tag{14e}$$

In agreement with the intuitive use of the words, the above equations describe the passive (14a), the active (14b), the binding or storage (14c), and the reflux (14d) components of transport respectively. Their physical significance is discussed in the following section.

Discussion.—In the preceding section we have presented a mathematical analysis which permits a complete and unambiguous categorization of transport processes occurring in living systems. This analysis is based on a description of the processes involved which is completely general and takes into account all forms of interaction between them. Although the particular form of description is that used in thermodynamics of irreversible processes [equations (1) and (2)], it is to be re-emphasized that we have not found it necessary to impose any such restrictions as linear relationships between fluxes and forces, reciprocal relations among phenomenological coefficients (i.e., the Onsager relations), or small deviations from equilibrium; thus the validity of our results is independent of these restricting conditions.

In order to clearly delineate those transport processes intuitively termed active, we have made use of the necessary condition that the only manner in which a chemical transformation can give rise to a transport process is by creating a source or a sink for the transported species. This condition is a direct consequence of Curie's theorem. Given the facts that the course of a chemical reaction is not associated with any direction in real space and is thus properly described by scalar quantities, while transport processes have directional properties, the applicability of the theorem and the validity of the conclusion are self-evident. It is thus possible to express the contribution of chemical reactions to the thermodynamic driving force of a given transport process in terms of the rate of the pertinent chemical reactions, as is done in equation (8).

Even though the contribution of metabolic reactions to a transport process must always take the form of a source or a sink, in a real system sources or sinks affecting the transport of a given component i may arise in more than one way and this has been explicitly taken into account. Thus, sources and sinks may influence the flux of a given component i either (1) by affecting the thermodynamic potential of i itself in unchanged chemical form or (2) by affecting the thermodynamic potential of a distinct constituent, k, which is derived from i by reversible chemical alteration for purposes of transport. Several types of such alterations, reversible upon completion of transport, are conceivable for a molecular species: (*a*) combination with another molecule, (*b*) removal of a chemical group, and (*c*) change of configuration. The contribution of chemical reactions to the transport of species i in any of such altered forms is explicitly taken into account by equation (11). The possibility that i may be transported in more than one altered form is expressed by summation over all possible k.

Similarly, the reactions affecting the thermodynamic potential of i itself may be further categorized into those in which (1) i is either put into or released from storage by a direct reaction, the product b not being transported, (2) i is altered to form a transportable species k and (3) i itself is not altered but its thermodynamic potential is affected by the production or removal of other constituents in the system. This categorization is made explicit by equation (12).

The total flux of any constituent i must of course include all contributions, whether produced by metabolism or not, as given by equations (13a) and (13b). These equations thus allow an unmistakable distinction between metabolically dependent and metabolically independent terms of the total flux. It is a matter of arbitrary convention whether the entire group of metabolically dependent terms, or only part of it is designated as "active." It appears to us, however, that a specific nomenclature for the individual flux terms separable on the basis of the origin of the thermodynamic force has the advantage of conceptual clarity. Accordingly, we have distinguished four terms: (1) the passive flux term (14a) which arises from forces to which metabolic processes make no direct contribution, (2) the active term (14b) describing the flux of i either in a chemically altered form or under the effect of metabolically dependent forces other than its own thermodynamic potential gradient, (3) the binding or storage term (14c) describing the flux of i resulting from the metabolically produced component of its own thermodynamic gradient, and (4) the reflux term (14d) which results from sources and sinks arising from the formation or decomposition of k, an altered transportable form of i. This last term represents in effect the flow of i in a direction opposite to its active flux in so far as this results from the changes of the chemical potential of i which accompany its transformation for purposes of transport.

Equations (14a) to (14e) provide the necessary framework within which the transport phenomena occurring in living systems may be completely described. However, to obtain their solution from experimental data one must have a sufficient number of simultaneous measurements of all the fluxes and forces occurring in the system to establish the functional relations between each of the coefficients and their forces. The number of required observations may of course be reduced by introducing simplifying assumptions whenever such may be applicable. For instance, an assumption that the coefficients λ_{ij} or $\lambda_{\alpha\epsilon}$ are constant will reduce the number of separate simultaneous observations of all fluxes and forces to $n + \rho$. Further simplifications result when it may be assumed that some of the λ_{ij}'s or $\lambda_{\alpha\epsilon}$'s vanish or that relations of the type $\lambda_{ij} = \lambda_{ji}$ and $\lambda_{\alpha\epsilon} = \lambda_{\epsilon\alpha}$ apply.

The above analysis indicates quite clearly that the question whether a given component i is transported actively or passively and to what extent cannot be given a precise and unique answer unless the system is completely described. The task of obtaining the answer is a formidable one and will require an approach by successive approximation.

Summary.—A general and inclusive formulation of transport processes in living systems is given and the conditions for the coupling of transport processes to chemical reactions are defined. It is shown that the contribution of metabolic reactions to the thermodynamic forces of transport can be described in terms of the reaction rates and that several different categories of transport processes may be distinguished on the basis of the origin of the thermodynamic forces involved. This allows a new and rigorous definition of active transport which is free from the ambiguities inherent in earlier attempts at its definition.

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[1] Rosenberg, T., *Acta Chem. Scand.*, **2**, 14 (1948).

[2] Rosenberg, T., *Symp. Soc. Exp. Biol.*, **8**, 27 (1954).

[3] Ussing, H. H., *Acta Physiol. Scand.*, **19**, 43 (1949).

[4] Hodgkin, A. L., and R. D. Keynes, *J. Physiol.*, **128**, 61 (1955).

[5] Ussing, H. H., *The Relation Between Active Ion Transport and Bioelectric Phenomena* (Rio de Janeiro: Instituto de Biofisica, Universidade de Brasil, 1955).

[6] Glynn, I. M., *J. Physiol.*, **134**, 278 (1956).

[7] Snell, F. M., "A Concept of Active Transport," in *Disturbance of Cellular Metabolism in the Premature Infant*, Report of the 32nd Ross Conference on Pediatric Research, ed. S. W. Fomon (Columbus: Ross Laboratories, 1959).

[8] Scheer, B. T., *Bull. Math. Biophys.*, **20**, 231 (1958).

[9] Jardetzky, O., *Science*, **125**, 931 (1957).

[10] Jardetzky, O., *Bull. Math. Biophys.*, **22**, 103 (1960).

[11] DeGroot, S. R., *Thermodynamics of Irreversible Processes* (New York: Interscience, 1952).

[12] Prigogine, I., *Introduction to Thermodynamics of Irreversible Processes* (Springfield, Ill.: C C Thomas, 1955).

[13] Curie, P., *Oeuvres* (Paris: Gauthier-Villars, 1908), p. 127.

[14] For systems continuous in space it should be taken as the change of the chemical potential gradient of i.

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*ENZYMIC MECHANISM OF INCREASED UTILIZATION OF GLUCOSE DURING VIRUS MULTIPLICATION IN THE CHORIOALLANTOIC MEMBRANE OF THE CHICK EMBRYO**

BY ERNEST KUN,† J. E. AYLING, AND BENJAMIN V. SIEGEL

DEPARTMENTS OF PHARMACOLOGY AND PATHOLOGY, UNIVERSITY OF CALIFORNIA SCHOOL OF MEDICINE, SAN FRANCISCO

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The chorioallantoic membrane of the chick embryo offers distinct advantages over other tissues for the study of multienzyme systems. Since it is composed of only three layers of cells, limiting factors of diffusion hardly interfere with measurements of the metabolism of intact cells *in vitro*. For these reasons we have undertaken the study of complex biochemical processes with the aid of excised chorioallantoic membranes. The influence of virus multiplication on the metabolism of the host tissue was one of the problems under investigation.

Earlier metabolic studies on the excised membrane revealed that during multiplication of myxoma,[1] fibroma, herpes simplex, vaccinia, Rous sarcoma, swine influenza, and Newcastle viruses,[2] the rate of lactic acid formation from glucose was markedly elevated. It was also shown that this increase in the accumulation of lactate was not due to inefficient transfer of electrons to O_2, an effect which might be assumed to occur as a consequence of "pathological changes caused by viruses," but was the result of an increased amount of glycolytic enzymes in cells where the multiplication of viruses took place. Aldolase and the glycolytic enzymes, catalyzing the formation of 3-phosphoglycerate from fructose 1-6-diphosphate were earlier identified[1, 2] and the increase of their concentration in infected membranes determined. It was found, furthermore, that the chorioallantoic membrane contains glucose-6-phosphate dehydrogenase.[3] This was believed to indicate that the dual

pathway of glucose metabolism (i.e., the "glycolytic" and "pentose cycle") may operate in this tissue. Analysis for both glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase revealed that the concentration of these enzymes were identical in normal and infected membranes. These observations resulted in the further study of the biochemical significance of increased glycolysis. An increase in the rate of the conversion of glucose to pyruvate would yield a net increase in the rate of ATP formation, which could presumably be made available for biosynthetic processes. It is expected that during virus multiplication there would be an increased rate of nucleic acid synthesis. Since the pentose constituents of nucleic acids arise from the direct oxidative pathway of hexose monophosphates, this pathway should operate at a higher rate when a "physiological demand" for pentose exists. Because no increase in the hexose-phosphate dehydrogenase content of infected membranes occurred, it was assumed that some other mechanism must be responsible for the activation of the oxidation of hexose monophosphates. It is subsequently shown that this mechanism is an increased rate of the oxidation of TPNH, mainly by pyruvate, catalyzed by lactate dehydrogenase. This enzyme is also synthesized at a higher rate in cells where the multiplication of viruses occurs.

Experimental.—Techniques used in the present studies were similar to the ones

TABLE 1

LACTIC ACID AND CO_2 PRODUCTION BY NORMAL AND INFECTED CHORIOALLANTOIC MEMBRANES

		Normal		Infected	
		Endogenous	+ Glucose*	Endogenous	+Glucose*
Aerobic	Q_{O_2}	0.34	0.31	0.30	0.43
	$Q_{lact.}$	0	0	0.56	1.13
Anaerobic	Q_{CO_2}	0.42	0.76	1.12	2.4
	$Q_{lact.}$	0.70	1.00	1.90	4.70

* 5 mg glucose per flask.
Q values = μmoles per 1 mg protein per 2 hr. Temperature = 30°C. Suspending fluid = 3 ml of allantoic fluid. Duration of experiment = 2 hr.

reported earlier.[1, 2] Chorioallantoic membranes of embryonated White Leghorn eggs, 12 days old, were infected with a suspension of a variant avian strain of canary pox virus. This virus was purified and identified by Siegel[4] and stored as a standard pool in ampules at −50°C throughout the experiments. The amount of virus used for each inoculum was 10^4 infectious particles per egg, as standardized by the method of pock counts.[4] It was shown earlier that inoculation with heat-inactivated viruses or extracts of chorioallantoic membranes free of viruses, had no effect on the metabolism of this tissue.[2] In most experiments biochemical measurements were made 48 hr after infection. The infected and control eggs were kept in an egg incubator at 36°C. The infected areas of the membranes were excised, cut into pieces of about $^1/_2$ cm^2 and suspended in a liquid medium, kept previously at 0°C, washed by transfer to fresh medium, and either used directly for manometric measurements or subjected to homogenization (in an all-glass homogenizer) and centrifugal cell fractionation[7] in 0.25 *M* sucrose, containing 9 mg cysteine hydrochloride per 10 ml sucrose (pH was adjusted to 7.4 with NaOH). The suspending medium in earlier studies was a buffered salt solution[1-3] but in the majority of recent experiments the centrifuged allantoic fluid or a salt solution[1] containing 400 mg bovine serum albumin per 100 ml was employed.

Manometric measurements were performed in conventional Warburg vessels at 30°C. Lactic acid was determined according to Barker and Summerson,[5] glucose by the method of Nelson,[6] and N by a modified Nessler procedure.[7] Glucose-1-C^{14} was a gift of Drs. E. Z. Hassid and E. F. Neufeld (Department of Biochemistry, Berkeley). The sample was purified by paper chromatography by Dr. Neufeld, and had a specific activity of 7.5 microcuries/mg. Analyses for C^{14} were done directly on filter paper strips (containing $K_2C^{14}O_3$) in a two-channel scintillation well counter by Dr. Chin-Tzu Peng in the Radioactivity Research Center of the School of Medicine. Crystalline heart muscle lactic dehydrogenase[8] was a gift from Dr. J. B. Neilands (Department of Biochemistry, Berkeley). All reagents were recrystallized from reagent grade sources; coenzymes were chromatographically purified samples, obtained from Sigma Chemical Co.

Results.—Manometric experiments on intact membranes: Recent experiments differed from earlier ones[1, 2] in 2 respects: (*a*) the suspending medium contained protein, and (*b*) the aerobic manometric measurements were done in 95% O_2 + 5% CO_2. Under these conditions pressure changes in the system are the result of both O_2 uptake and CO_2 evolution. Anaerobic production of CO_2 was determined under the same conditions with 95% N_2 + 5% CO_2. It was found that under aerobic conditions the normal membrane, suspended in allantoid fluid containing glucose, produces only negligible amounts or no lactic acid at all. On the other hand, 48 hr after virus inoculation, during a period of exponential viral multiplication,[9] aerobic lactate accumulation does occur. Under anaerobic conditions the infected membranes evolve much larger amounts of lactate and CO_2 than the normal ones. A representative experiment is shown in Table 1.

TABLE 2

GLUCOSE UPTAKE AND LACTATE FORMATION BY CHORIOALLANTOIC MEMBRANES

		Normal	Infected
Aerobic	Q_{O_2}	0.72	0.62
	$Q_{glucose}$	0.34	0.70
	$Q_{lact.}$	0.39	0.87
Anaerobic	Q_{CO_2}	0.55	0.92
	$Q_{glucose}$	0.60	1.20
	$Q_{lact.}$	2.3	3.46

Time of experiment = 5 hr. Temperature = 30°C. Suspending medium = buffered salt solution containing albumin. Q values = μmoles per 1 mg protein.

The rate of lactic acid formation exceeded the amount of CO_2 evolved, suggesting that the mechanism by which lactate is produced does not involve a marked change in the total H^+ concentration. It is of interest to note that the use of protein containing suspending medium, as suggested by Warburg[10] abolishes aerobic lactate accumulation in normal membranes. This indicates the susceptibility of electron transferring enzymes in intact cells to denaturation by relatively mild agents, e.g., salts.

The question arises whether or not the increase of lactate accumulation occurs simultaneously with a net increase in glucose consumption. The experimental answer to this question is complicated by the fact that intact membranes as well as allantoic fluid contain variable amounts of endogenous carbohydrate. The interference of endogenous sources of glucose can be diminished by the use of albumin containing suspending medium, and by prolonged incubation, when larger dif-

ferences in glucose utilization become measurable by analyses for glucose in the suspending medium. Although the accurate determination of glucose utilization is not possible, the large increase in glucose disappearance is a fair indication that increased lactate accumulation occurs simultaneously with increased glucose consumption. This is shown in Table 2. There is aerobic lactic acid production by the normal membranes suggesting that prolonged incubation in the albumin containing salt solution interferes with the efficient aerobic utilization of pyruvate.

The pathway of glucose utilization was further studied with the aid of C^{14} labeled glucose in the 1 position. The conditions were the same as described for the experiment summarized in Figure 1, except that each flask contained 2 microcuries of glucose-1-C^{14} as a tracer. The gas phase was either 100% O_2 or 100% N_2. The reaction was stopped by tipping into the main compartment 0.3 ml 2 *N* HCl, which lowered the pH below 2.0. The CO_2 (containing $C^{14}O_2$) was absorbed on a filter-

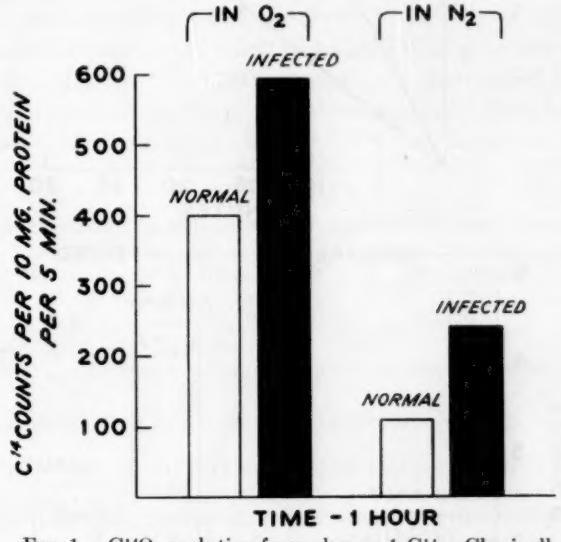

FIG. 1.—$C^{14}O_2$ evolution from glucose-1-C^{14}. Chorioallantoic membranes were incubated in bicarbonate-free buffered salt solution containing serum albumin in the presence of 5 mg glucose and glucose-1-C^{14} as tracer. Details are described in the text under *Experimental*.

paper strip saturated with 10 *N* KOH, placed into the center well. Results are summarized in Figure 1. The rate of $C^{14}O_2$ evolution is much greater under aerobic conditions, when both anaerobic and aerobic metabolic pathways operate. Under these conditions $C_1{}^{14}O_2$ derived from oxidative decarboxylation of 6-phosphogluconate is in part further metabolized by the tricarboxylic acid cycle. The increase in $C^{14}O_2$ formation during virus multiplication was 47.5 per cent. The rate of $C^{14}O_2$ evolution from $C_1{}^{14}$-glucose under anaerobic conditions is more closely a measure of the rate of decarboxylation of 6-phosphogluconate since only the anaerobic CO_2 fixing enzymic reactions interfere with the detection of the formation of $C^{14}O_2$. The increase in $C^{14}O_2$ evolution by membranes supporting virus multiplication under N_2 atmosphere was 120 per cent. It is probable that the increased rate of hexose-6-phosphate oxidation varies between these extreme values (50 to

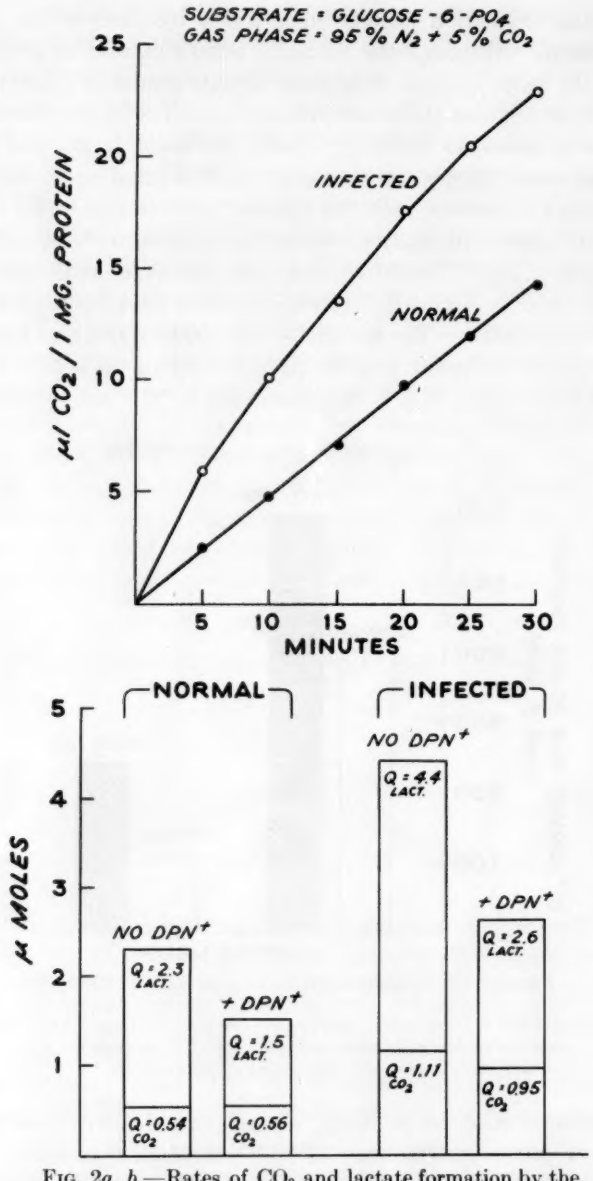

FIG. 2*a*, *b*.—Rates of CO_2 and lactate formation by the soluble cytoplasmic fraction of homogenates of chlorioallantoic membranes were determined in the manometric test system, described in the text under *Experimental*.

In Fig. 2*a* the CO_2 production is illustrated as a function of time, while in Fig. 2*b* the effect of added DPN^+ (2.5 μmoles) on CO_2 and lactate formation is summarized. Q values = μmoles per 1 mg protein per 30 min.

120%) in all membranes where multiplication of viruses takes place. Metabolic measurements on intact membranes thus revealed that both glycolysis and the hexose monophosphate shunt operate at a higher rate during active virus reproduction.

The effects observed with intact membranes were reproduced in cell-free systems. It was found that the centrifugal supernatant of sucrose homogenates (24.000 × *g* for 30 min) contained the whole enzyme system necessary for the demonstration of the virus-induced increase in CO_2 and lactate production. Some component of the system was found to be sensitive to oxidation by atmospheric O_2 as manifested in a progressive diminution of the virus effect upon standing. This instability was counteracted by the use of cysteine in the homogenizing medium. The cell-free system contained 30 μmoles of glucose-6-phosphate (K-salt), 2.5 μmoles of TPN^+, 10 μmoles of $MgSO_4$, 20 μmoles of Na-pyruvate and 80 μmoles of nicotinamide, 30 μmoles of PO_4, 30 μmoles of HCO_3, and 9 mg protein (supernatant of a homogenate, prepared in 0.25 *M* sucrose, containing cysteine). The volume of the reaction mixture was made up to 3.1 ml with sucrose-cysteine solution, and the reaction measured for 30 min under 95% N_2 + 5% CO_2. Since under these conditions it is expected that both the direct oxidation of glucose-6-phosphate and, due to endogenous adenine nucleotides and traces of DPN^+, its fermentation to lactate take place simultaneously, the effect of added DPN^+ was also studied. It was found that addition of DPN^+ (2.5 μmoles) markedly diminished lactate accumulation but not CO_2 production in the preparations obtained either from normal or infected membranes. This inhibitory effect of DPN^+ on lactate accumulation argues

TABLE 3

EFFECT OF IODOACETATE ON CO_2 AND LACTATE FORMATION BY THE SUPERNATANT FRACTION

	Normal		Virus Infected	
	No inhibitor	Iodoacetate*	No inhibitor	Iodoacetate*
Q_{CO_2}	0.77	0.49	1.1	0.42
$Q_{lact.}$	2.0	0.95	4.9	0.84

* Iodoacetate = 6.7×10^{-3} *M*.
Time of incubation = 30 min. Q values = μmoles per 1 mg protein per 30 min. Substrate = glucose-6-phosphate.

against the assumption that the formation of lactate is solely due to the reaction

$$\text{pyruvate} + DPNH^+ + H^+ \rightarrow \text{lactate} + DPN^+.$$

Attempts to eliminate the interference of endogenous nucleotides by adsorption techniques were only partially successful and the susceptibility of the system to oxidation during these procedures led us to abandon this effort. The effects of added DPN^+, and of virus infection on the rate of CO_2 and lactate formation are shown in Figure 2*a*, *b*. The "protection" by cysteine indicated that a thiol system is involved in this multienzyme system. The effect of iodoacetate was therefore determined. As shown in Table 3, a concentration of iodoacetate which completely inhibits triose phosphate dehydrogenase only partially blocks these reactions, again suggesting that an additional mechanism besides glycolysis is in part responsible for the accumulation of lactate.

Analyses of the condition of these reactions revealed that the mechanism of "nonglycolytic" lactate formation is

$$TPNH + H^+ + \text{pyruvate} \rightarrow \text{lactate} + TPN^+.$$

The enzyme responsible for this reaction was found to be a lactate dehydrogenase (LHD) of the chorioallantoic membrane. The following observations support this conclusion:

(*a*) no evidence was found for a direct transfer of H between DPN^+ and TPN^+ in the soluble extract of this tissue;
(*b*) there is a parallel increase of LDH concentration with increased CO_2 and lactate formation;
(*c*) this LDH catalyzes the reduction of pyruvate by both DPNH and TPNH;
(*d*) DPN^+ in a concentration range of 10^{-4} to 10^{-5} *M* almost completely inhibits the TPNH-pyruvate reaction catalyzed by either crystalline heart muscle enzyme[8] or the chorioallantoid LDH;
(*e*) the chorioallantoic LDH is susceptible to inhibition by iodoacetate when it catalyzes the reaction $TPNH + H^+ +$ pyruvate $\rightarrow$ lactate $+ TPN^+$. The DPNH $\rightarrow$ pyruvate reaction is not inhibited by iodoacetate.

A substrate mediated transhydrogenation of the type suggested by Holzer[11] is quite improbable because of the inhibition of the TPNH-pyruvate reaction by DPN^+. It should also be noted that Holzer used 0.4 mg of crystalline LDH and 0.48 mg glucose-6-phosphate dehydrogenase (as TPNH generator) to produce an estimated absorbancy change at 366 mμ (presumably due to TPNH) of 0.120 in 22 to 58 min. These conditions do not seem to support strongly the suggestion that LDH can catalyze a substrate mediated transhydrogenation to a significant extent.

TABLE 4

LDH CONTENT OF CHORIOALLANTOIC MEMBRANES AFTER INOCULATION WITH AVIAN POX VIRUS

Time	Normal	Infected
6 hours	43	53
24 "	23	57
30 "	43	97
48 "	30	77
72 "	54	73
78 "	32	77

The results are expressed in micromoles $\times 10^2$ of DPNH oxidized per 1 mg protein per 1 min. The optical system contained 30 μmoles of Na-pyruvate 0.4 to 0.6 μmoles of DPNH, 250 μmoles of phosphate (pH 7.4) in a volume of 3 ml. The amount of protein (supernatant fraction) varied between 5 and 12 gamma per cuvette. Absorbancy changes were read at 30 sec intervals for 4 min.

It is of interest that neither the DPNH-pyruvate nor TPNH-pyruvate reactions catalyzed by heart muscle LDH are inhibited by iodoacetate in concentrations employed here. A detailed kinetic study of these differences will be dealt with elsewhere.

The increase in LDH content of chorioallantoic membranes, supporting the multiplication of avian pox virus, is shown in Table 4. The effect of iodoacetate on the oxidation of DPNH and TPNH by pyruvate and LDH of the chorioallantoic membrane and of heart muscle are summarized in Table 5.

It was of interest to determine whether or not the amount of other pyridine nucleotide dehydrogenases is changed when the membrane supports virus multiplication. The following enzymes were tested in the supernatant fraction: malic enzyme, malic dehydrogenase, glutamic dehydrogenase, and glutathione reductase. Results are shown in Table 6. A marked increase in malic dehydrogenase and GSSG reductase occurred 48 hr after virus infection. The glutamic dehydrogenase content of this tissue is low and the significance of the increase of this enzyme is questionable. On the other hand, the oxidation of TPNH by either GSSG or oxalacetate may be of importance. Since there is at present no experimental evidence indicating an increased rate of oxalacetate formation after virus infection

while the observed increase in glycolysis yields a higher rate of pyruvate production, it seems likely that the oxidation of TPNH by pyruvate has priority in the mechanism of increased glucose utilization. The role of GSSG reductase is probably a significant one, but at this time difficult to explain.

Discussion.—According to earlier experiments[1, 2] and the ones reported here, the chorioallantoic membrane responds to virus infection with a marked increase in glycolytic enzymes. Similar observations were made recently with poliomyelitis virus on tissue cultures of epithelium cells.[14] It is probable that the primary event in virus infection, in a metabolic sense, is a specific influence of a virus component on the enzyme-synthesizing system, which responds by a hitherto-unknown mechanism with an activation of synthesis of certain enzyme proteins. As shown here, the increase of the concentration of certain enzyme proteins can profoundly modify the metabolic pattern of the "infected" cell. It seems reasonable to think that this modification of metabolism will eventually result in the resynthesis of the constituents of the virus itself. We have so far only studied certain phases of carbohydrate metabolism and the example for the hypothesis offered above is restricted to the increased formation of the pentose constituent of virus nucleic acid.

Many challenging questions arise. Calculations based on cell counts of chorio-

TABLE 5

THE EFFECT OF IODOACETATE ON THE ENZYMIC OXIDATION OF DPNH AND TPNH

	DPNH				TPNH			
	Normal		Infected		Normal		Infected	
	O	Iodoac.	O	Iodoac.	O	Iodoac.	O	Iodoac.
Chorioallantois enzyme	32	34	79	77	4	1.6	6.5	2.3
Heart muscle LDH (cryst.)	7,000	7,000	..	..	1,900	1,900	..	..

Results are expressed as in Table 4. The concentration of TPNH was 1 micromole per 3 ml iodoacetate concentration = 1.4×10^{-2} *M*.

The centrifugal supernatant (chorioallantois enzyme) was tested 48 hr after inoculation with the virus. The amount of heart muscle LDH used in the optical test was 0.05 to 0.1 gamma protein.

allantoic membranes and titration of infective particles 48 hr after inoculation reveal that the number of cells containing infective viruses is only 10 to 20 per cent of the total cell population studied. An increase of 50 per cent in the average concentration of certain enzymes in the whole tissue may mean an increase of several hundred per cent in the infected cell or it may reflect an effect even in the cells which do not contribute to virus multiplication. There is no answer as yet to this question.

The virus-induced onset of aerobic lactate accumulation in this apparently "aerobic" tissue suggests, according to Warburg, that in appearance the membrane exhibits characteristics of a cancerous tissue. While a generalization is as yet not timely, this observation suggests to us a reinvestigation of certain aspects of glucose metabolism of cancer cells and a search for the type of mechanism of lactate accumulation as found in virus-infected membranes. It is obvious that the increase in concentration of certain enzymes does not have to be excessive in order to bring about profound metabolic changes. Again, the appearance of glycolytic enzymes in the serum of tumor-bearing animals, as found by Warburg[15] and confirmed by Sibley and Lehninger,[16] may be an indication of their increased biosynthesis.

The change in glucose utilization by the chorioallantoic membrane is readily

explained by an increased concentration of glycolytic enzymes. Lactic acid dehydrogenase plays a special role since it effects an oxidation of TPNH by pyruvate and causes an increased turnover in the "pentose cycle." Our results on the catalytic role of LDH in TPNH oxidation are in close agreement with those of Navazio, Ernster and Ernster.[17] The striking inhibition of the TPNH → pyruvate reaction by physiological concentrations of DPN^+, also observed by these authors, may have a physiological significance. It is noteworthy that TPN^+ has no inhibitory effect at all on the DPNH → pyruvate reaction. This implies that the oxidation of TPNH by pyruvate, catalyzed by LDH would only be possible when the amount of available DPN^+ in the cell is very low. The actual concentration of DPN^+ in, for example, normal rat liver is about 0.6–0.7 μmoles per gm,[18] sufficient to cause a marked inhibition. It is quite possible that a change in the intracellular distribution of DPN^+, in addition to the rate of its reduction, constitutes regulatory mechanisms which contribute to the control of the rate of oxidation of TPNH by pyruvate.

It is of considerable interest that the isotope experiments of Wenner *et al.*[19] showed that in Novikoff hepatoma and ascites tumor cells the major oxidizing agent of TPNH is pyruvate. It seems likely that the enzymic mechanism of TPNH oxi-

TABLE 6

CONCENTRATION OF DEHYDROGENASES IN THE CHORIOALLANTOIS 48 HR AFTER VIRUS INOCULATION

	Normal		Infected	
Enzyme	DPNH	TPNH	DPNH	TPNH
Malic enzyme	...	0.8	...	0.8
Malic dehydrogenase	29.0	4.3	53.0	7.3
Glutamic dehydrogenase	0.2	...	0.3	...
GSSG reductase	...	3.9	...	5.7

Results are expressed as in Tables 4 and 5. Malic enzyme and glutamic dehydrogenase were assayed according to standard procedures,[12] malic dehydrogenase, as described elsewhere,[13] and glutathione (GSSG) reductase by the oxidation of TPNH (1 μmole) in the presence of 15 moles of GSSG at pH 7.4 in 0.1 *M* phosphate buffer in a volume of 3 ml.

dation in these tumor cells may be the same as in the virus-infected chorioallantoic membrane.

The LDH of the chorioallantoic membrane differs from enzymes of other sources in several respects. The most striking difference is the iodoacetate sensitivity of the TPNH → pyruvate reaction. None of the other reactions catalyzed by this LDH are inhibited by iodoacetate. The heart muscle enzyme[8] is quite refractory to thiol reagents other than *p*-chloromercuribenzoate. Ox heart LDH contains 7 to 9 SH groups per mole, but only 3 to 4 of these react with *p*-chloromercuribenzoate in the native protein.[20, 21] The SH groups of heart muscle LDH probably participate in the binding of coenzymes to the protein.[21] The significance of the thiol sensitivity of the LDH of the chorioallantois, in the binding of coenzymes, and their analogues, may become better understood when kinetic studies similar to those reported by Kaplan *et al.*[22] have been completed.

Studies dealing with the LDH and malic dehydrogenase content of the chick embryo during aging have been reported by Solomon.[23] LDH activity on a protein basis in organs of the embryo remain constant except in the heart, where a small increase occurs on the 14th day. In our experiments, the analytical results of virus-infected membranes were in each instance compared with normal embryos

of identical age. Therefore, slight variations caused by aging were constantly compensated for.

Summary.—(1) Avian pox virus-infected chorioallantoic membranes of chick embryos, suspended in allantoic fluid, produce lactic acid from glucose under aerobic conditions. Under identical conditions, non-infected membranes do not accumulate lactate.

(2) The rates of glucose consumption and lactate and CO_2 production (the latter two under anaerobic conditions) is markedly increased as the result of virus infection.

(3) The rate of CO_2 formation from $C_1{}^{14}$-labeled glucose is increased between 50 to 120 per cent 48 hours after inoculation with virus.

(4) The same effect of virus infection was reproduced with a soluble enzyme preparation (centrifugal supernatant) and it was shown that virus infection causes an increased synthesis of glycolytic enzymes resulting in an increased rate of pyruvate formation. LDH of the membrane catalyzes the oxidation of TPNH by pyruvate. The increase in LDH after virus infection accounts for the increased operation of the hexose monophosphate shunt in intact membranes.

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[1] Kun, E., and M. H. D. Smith, *Proc. Soc. Exp. Biol. & Med.*, **73**, 628 (1950).

[2] Smith, M. H. D., and E. Kun, *Brit. J. Exp. Path.*, **34**, 1 (1954).

[3] Kun, E., *Proc. Soc. Exp. Biol. & Med.*, **83**, 532 (1953).

[4] Siegel, B. V., *Virology*, **2**, 356 (1956).

[5] Barker, J. B., and W. H. Summerson, *J. Biol. Chem.*, **138**, 535 (1941).

[6] Nelson, N., *J. Biol. Chem.*, **153**, 375 (1944).

[7] Umbreit, W. W., R. H. Burris and J. F. Stauffer, *Manometric Techniques* (Minneapolis: Burgess Publ., 1957), p. 188.

[8] Neilands, J. B., *J. Biol. Chem.*, **208**, 225 (1954).

[9] Siegel, B. V., and A. A. Batts, *Arch. ges. Virusforsch.*, **7**, 498 (1957).

[10] Warburg, O., *The Metabolism of Tumors* (London: Constable Co., 1930).

[11] Holzer, H., and S. Schneider, *Biochem. Z.*, **330**, 240 (1958).

[12] Colowick, S., and N. O. Kaplan, eds., *Methods in Enzymology* (New York: Acad. Press, 1955), vol. 1, p. 748; vol. 2, p. 220.

[13] Davies, D. D., and E. Kun, *Biochem. J.*, **66**, 307 (1957).

[14] Matzelt, D., and J. Homan, *Biochem. Z.*, **330**, 245 (1948); *ibid.*, 260 (1958).

[15] Warburg, O. and W. Christian, *Biochem. Z.*, **314**, 399 (1943).

[16] Sibley, J. A., and A. L. Lehninger, *J. Biol. Chem.*, **177**, 859 (1949); *J. Nat. Cancer Inst.*, **9**, 303 (1948).

[17] Navazio, F., B. B. Ernster and L. Ernster, *Biochim. Biophys. Acta*, **26**, 416 (1957).

[18] Dickens, F., E. G. Glock and P. McLean, in *Regulation of Cell Metabolism*, Ciba Foundation Symp., ed. G. E. W. Wolstenholm and C. O'Conner (Boston: Little Brown & Co., 1959), p. 176.

[19] Wenner, C. E., J. H. Hackney, and F. Moliterno, *Cancer Res.*, **18**, 1105 (1958); Wenner, C. E., *J. Biol. Chem.*, **234**, 2472 (1959).

[20] Nygaard, H. P., *Acta Chem. Scand.*, **10**, 397 (1956).

[21] Takenaka, Y., and G. W. Schwert, *J. Biol. Chem.*, **223**, 157 (1956).

[22] Kaplan, N. O., M. M. Ciotti, M. Hamolsky and R. E. Bieber, *Science*, **131**, 392 (1960).

[23] Solomon, J. B., *Biochem. J.*, **70**, 529 (1958).

THE SYNTHESIS OF 5-METHYLURIDINE 5'-PHOSPHATE IN RAT EMBRYO EXTRACTS*

By Frank Maley

DIVISION OF LABORATORIES AND RESEARCH, NEW YORK STATE DEPARTMENT OF HEALTH, ALBANY

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The occurrence of 5-methyluridine in bacterial ribonucleic acid[1] and in rat liver slices incubated with thymine[2] raises the problem of how this nucleoside and its possible derivatives are synthesized. One pathway apparently involves the reaction of ribose-1-phosphate and thymine in the presence of a nucleoside phosphorylase that has been found in bacterial[3] and animal tissue extracts.[4, 5] Another pathway, which will be described in this paper, involves the methylation of UMP† to 5-methyl UMP by extracts of rat embryos. This reaction appears to be analogous to that described for the thymidylate synthetase reaction,[6] for it requires the presence of THFA and a one-carbon donor. However, it is as yet undetermined whether the same enzyme system is involved.

Materials and Methods.—Most of the nucleotides employed were purchased from the Sigma Chemical Company, St. Louis, Missouri. Deoxy-UMP and HMU were prepared by the nitrous acid deamination of *d*-CMP and HMC, respectively. HMC was purchased from the California Corporation for Biochemical Research, Los Angeles, California. MUR and HMUR were synthesized by the procedure of Cline *et al.*[7] THFA was prepared by the reduction of folic acid as described by Blakley[8] and an extinction coefficient of 2.8×10^3 per mole was employed.[9] UMP-2-C^{14} was chemically prepared from UR-2-C^{14}.[10] C^{14}-formaldehyde was purchased from the Volk Radiochemical Company, Chicago, Illinois. Radioactivity was determined with an end window gas flow counter for a period of time calculated to give ± 5 per cent standard deviation. Rattlesnake venom (*Crotalus adamanteus*) was purchased from the Ross Allen Reptile Institute, Silver Springs, Florida.

Radioactive compounds were isolated as follows: The supernatant from each reaction mixture was incubated with 1 mg of snake venom for 30 minutes at 37°C. After the addition of 0.2 ml of 0.2 *M* sodium bisulfite, the incubation mixture was passed through a column of Dowex 1-formate on top of a column of Dowex 50-H+ (each column was 1 cm wide by 3 cm long). The column was washed with water until a volume of 15 ml was collected. All of the unreacted formaldehyde appeared to be retained by the column as the THFA or bisulfite complex. An appropriate aliquot (0.5 ml) was plated for counting. The remaining eluate was concentrated to less than 2 ml, placed in a conical centrifuge tube, and lyophilized. To isolate the pyrimidine bases, the lyophilized residue was hydrolyzed at 100°C with 72 per cent perchloric acid for 1 hour, 0.2 ml water added, and an aliquot (0.05 ml) chromatographed on Whatman 3MM paper with either isopropanol-HCl[11] or *n*-butanol.[12] The nucleosides were isolated by dissolving the lyophilized residue in 0.2 ml of water and chromatographing an aliquot (0.05–0.1 ml) on Whatman 3MM paper with secondary butanol.[7] For the preparation of radioautograms, 0.05 ml of the solution was placed in the corner of a sheet of Whatman No. 1 paper (35 cm × 30 cm) and chromatographed with secondary butanol (ascending) in the first dimension and ethyl acetate, formic acid, water[2] or *t*-butanol, methylethyl

ketone, water, HCl[7] in the second dimension (also ascending). Kodak Blue Brand X-ray film (10 × 11¾ in.) was employed for the radioautograms with an exposure time of 1–2 weeks.

Results.—As can be seen in Table 1, C^{14}-formaldehyde fixation requires the presence of UMP, *d*-UMP, or *d*-CMP. Similar to the results obtained by others with *d*-UMP,[6, 13] UMP is required for the incorporation of formaldehyde, a reaction also found to be effected by chick embryo and *Escherichia coli* extracts.[14] UR was found to be ineffective in promoting this reaction. The occurrence of appreciable radioactivity in the blanks has not been satisfactorily explained as yet. Some, but not all, of this appears to be due to endogenous nucleotides. Serine has been eliminated as a possibility, since C^{14}-serine could not be detected in the eluate when passed through the column. However, the possible reaction with amines has not been eliminated. With shorter incubation periods, 10–20 minutes, the blanks are not appreciable and good linearity is obtained in plotting counts fixed versus concentration of extract.

TABLE 1

NUCLEOTIDE DEPENDENCE OF C^{14}–FORMALDEHYDE FIXATION

Experiment	Compound added	μMoles added	Cpm fixed
I	O		6,864
	d-UMP	8.80	62,128
	UMP	8.20	23,584
II	O		745
	UMP	4.35	7,140
	d-UMP	4.20	26,600
	d-CMP	9.00	27,100

Experiment I. In addition to the added nucleotides listed in the Table, each reaction mixture contained the following components in μMoles: $MgCl_2$, 10; Tris buffer (pH 7.98), 200; C^{14}–formaldehyde, 6.0 (2.82 × 10^5 cpm/μMoles) THFA, 9; BAL, 1.7; 0.5 ml of the supernatant fraction of a 50 per cent isotonic KCl homogenate of 14–16 day rat embryos. The total volume was 2.0 ml. After incubation at 37°C for 2 hr, the reaction was stopped by heat. The isolation of the radioactive components is described under *Materials and Methods*.

Experiment II. Treated the same as Expt. I except for the incubation time, which was 1 hr. The reaction mixture contained the following components in μMoles: $MgCl_2$, 10, sodium phosphate buffer (pH 7.5), 100; THFA, 3.6; BAL, 0.66; C^{14}-formaldehyde, 1.4 (2.82 × 10^5), cpm/μMole, and 0.4 ml of the supernatant fraction of an isotonic KCl homogenate of rat embryo. The total volume was 1.0 ml.

Deoxy-CMP incorporates formaldehyde by virtue of its rapid conversion to *d*-UMP.[15, 16] This deamination is very effective in rat embryos as demonstrated by the similarity in counts fixed between *d*-CMP and *d*-UMP (Table 1). The incorporation into UMP is only about one-fourth as effective, but the distribution of counts among the nucleosides isolated by chromatography is usually more varied. As shown in Table 2, most of the counts are distributed between the UR and the MUR regions. The appearance of radioactivity in the UR region is not due to tailing, as the secondary butanol system is quite efficient in separating UR, MUR, and TDR. The cause for this radioactivity, which has not been obtained in some experiments, will be discussed below. Markers of MUR and TDR aided in distinguishing the desired compounds, which were cut out from the chromatogram, eluted, and counted.

Identification of 5-Methyluridine.—1. *Isolation of thymine:* Proof that the formaldehyde fixed in the presence of UMP is due to the formation of a thymine-containing compound was obtained by hydrolysis of the lyophilized column eluate with perchloric acid to obtain the pyrimidine bases. In a typical experiment 15,070 cpm were obtained in the column eluate from a reaction mixture containing UMP. After this eluate was treated as described under *Materials and Methods*

and chromatographed in secondary butanol, 11,700 cpm were eluted from the MUR region. The eluate from an identical reaction mixture, when subjected to perchloric acid hydrolysis and chromatography in isopropanol–HCl,[11] and *n*-butanol[12] yielded 10,145 and 9,624 cpm in the thymine regions of these chromatograms. Similarly, isolation of the MUR region by secondary butanol chromatography, followed by perchloric acid hydrolysis and rechromatography, revealed that almost all of the counts migrated with thymine.

2. *Isolation of MUR by electrophoresis:* On electrophoresis in 1 per cent sodium tetraborate, as previously described,[16, 17] chemically prepared MUR migrated more slowly than UR. After 130 minutes, UR moved 10.1 cm toward the anode, and MUR, 8.0 cm. In one experiment, the UMP remaining in the reaction mixture was isolated by gradient elution chromatography.[18] This region should also contain 5-methyl UMP, if present. The isolated UMP was converted to UR with snake venom, a carrier amount of MUR added, and the mixture subjected to electrophoresis. Almost all of the radioactivity was present in the MUR region. In another experiment, the reaction mixture was scaled up to five times the amount used in Experiment II. After secondary butanol chromatography, the ultraviolet-light-absorbing region corresponding to MUR was removed and eluted. The

TABLE 2

SEPARATION OF RADIOACTIVE COMPOUNDS FROM INCUBATION MIXTURES OF EXPERIMENTS I AND II BY CHROMATOGRAPHY IN SECONDARY BUTANOL[7]

Experiment	Chromatographic Region	Initial Compound Added: O cpm	UMP cpm	*d*-UMP cpm
I	Uridine	570	6,516	986
	5-Methyluridine	637	10,704	1,292
	Thymidine	612	392	48,000
II	Uridine	30	3,354	42
	5-Methyluridine	102	2,274	1,866
	Thymidine	30	174	15,114

280/260, 250/260 ratios were 0.42 and 0.83, respectively, which do not agree with the listed ratios for MUR.[7, 19] Since uracil has the same Rf as MUR, contamination by this compound would account for the deviation. On electrophoresis of the above MUR region, two ultraviolet-light-absorbing regions were obtained, one corresponding in migration to uracil and the other to MUR. The 280/260, 250/260 ratios of the isolated compounds, 0.26, 0.88 for the former and 0.70, 0.65 for the latter, agree well with listed ratios for uracil and MUR.

3. *Two-dimensional radioautography:*‡ Two-dimensional chromatography followed by exposure of the X-ray film to the chromatogram, as presented under *Materials and Methods,* showed that the reaction was more complex than originally expected from the data obtained in Table 2. In addition to the radioactive spot which coincided exactly with the ultraviolet-light-absorbing area of added carrier MUR, a number of other radioactive areas were obtained. One of these appears to be due to HMU, which would account for the radioactivity in the UR region of Table 2. Another appears to be due to HMUR. These areas showed up even more clearly when UMP-2-C^{14} was employed in the incubation mixture with an excess of unlabeled formaldehyde. In this instance, electrophoresis was used to separate the nucleosides and pyrimidines. The latter were eluted from a region

near the origin and subjected to two-dimensional chromatography (secondary butanol in the first dimension and *t*-butanol, methylethyl ketone, HCl, water[7] in the second dimension). Two radioactive spots were obtained with the appropriate Rf's for uracil and HMU.[7] As stated before, in some instances only trace amounts of radioactivity were obtained in the UR region. It appears that this may reflect the amount of nucleoside phosphorylase present, which in turn could be determined by the age of the embryos used as an enzyme source. However, further studies are necessary in order to clarify this point and to identify the other radioactive areas.

The two-dimensional radioautograms of the reaction mixtures containing *d*-UMP are similarly complex, but the intensity of the radioactive spots other than TDR and thymine is minor in comparison. One spot suggests the presence of hydroxymethyldeoxyuridine. This is in accord with the finding of hydroxymethyl *d*-UMP in extracts of *E. coli* during studies on thymidylate synthetase.[20]

Discussion.—The formation of 5-methyl UMP in embryonic tissue and possibly in all tissues containing measurable thymidylate synthetase activity (neoplasms, thymus, regenerating liver, bacteria, etc.) has many obvious implications. One deserving immediate consideration is the possible incorporation of this compound into the ribonucleic acid of animal tissues. Though 5-methyl UMP has been detected in the ribonucleic acid of bacteria,[1] it has not been found in animal tissues as yet. Perhaps a more thorough investigation is warranted, particularly of those tissues capable of forming 5-methyl UMP. Reichard[21] has reported that MUR is only slightly incorporated into the ribonucleic acid of regenerating liver, but due to the poor phosphorylation of this compound[5] to the nucleotide level, this result could have been anticipated. However, this obstacle is bypassed in those tissues capable of forming 5-methyl UMP directly from UMP.

The finding of hydroxymethyl derivatives of UMP and possibly of *d*-UMP raises the possibility that these compounds are intermediates in the formation of 5-methyl UMP and TMP. Flaks and Cohen[20] have found that C^{14} labeled 5-hydroxymethyl *d*-UMP is not converted to TMP in bacterial extracts. Similar results have been obtained in this laboratory with embryo extracts. Despite these results such a role has not been ruled out for 5-hydroxymethyl UMP. Further studies will be necessary to clarify this point.

Summary.—A method for estimating the methylation of UMP and *d*-UMP in tissue extracts is presented. By this procedure it has been demonstrated that UMP is converted to 5-methyl UMP in extracts of rat embryos. This reaction requires the presence of THFA and formaldehyde and appears to be accompanied by intermediates or side products, as revealed by radioautography.

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† The following abbreviations are used: THFA, tetrahydrofolate; Tris, tris (hydroxymethyl) aminomethane; BAL, 2,3-dimercaptopropionate; UR, uridine; MUR, 5-methyluridine; HMUR, 5-hydroxymethyluridine; TDR, thymidine; HMU, 5-hydroxymethyluracil; HMC, 5-hydroxymethylcytosine; UMP, uridine 5′-phosphate; TMP, thymidine 5′-phosphate; CMP, cytidine 5′-phosphate; *d*-, deoxy.

‡ A more detailed account of these studies is in preparation.

[1] Littlefield, J. W., and D. B. Dunn, *Biochem. J.*, **70**, 642 (1958).

[2] Fink, K., R. E. Cline, R. B. Henderson, and R. M. Fink, *J. Biol. Chem.*, **221**, 425 (1956).

[3] Lampen, J. O. in *Phosphorus Metabolism*, ed. W. D. McElroy and B. Glass (Baltimore: The Johns Hopkins Press, 1952), vol. 2, p. 368.
[4] Friedkin, M., and D. Roberts, *J. Biol. Chem.*, **207**, 257 (1954).
[5] Potter, V. R., and C. deVerdier, *J. Nat. Cancer Inst.*, **24**, 13 (1960).
[6] Friedkin, M., and A. Kornberg, in *The Chemical Basis of Heredity*, ed. W. D. McElroy and B. Glass (Baltimore: The Johns Hopkins Press, 1957), p. 609.
[7] Cline, R. E., R. M. Fink, and K. Fink, *J. Am. Chem. Soc.*, **81**, 2521 (1959).
[8] Blakley, R. L., *Biochem. J.*, **65**, 331 (1957).
[9] Blakley, R. L., *Nature*, **182**, 1719 (1958).
[10] Michelson, A. M., *J. Chem. Soc.*, 1957 (1958).
[11] Wyatt, G. R., *Biochem. J.*, **48**, 584 (1951).
[12] Markham, R., and J. D. Smith, *Biochem. J.*, **45**, 294 (1949).
[13] Humphreys, G. K., and D. M. Greenberg, *Arch. Biochem. and Biophys.*, **78**, 275 (1958).
[14] Maley, F., unpublished observations.
[15] Scarano, E., *Biochim. Biophys. Acta*, **29**, 459 (1958).
[16] Maley, G. F., and F. Maley, *J. Biol. Chem.*, **234**, 2975 (1959).
[17] Maley, F., and G. F. Maley, *Biochim. Biophys. Acta*, **31**, 577 (1959).
[18] Hurlbert, R. B., H. Schmitz, A. F. Brumm, and V. R. Potter, *J. Biol. Chem.*, **209**, 23 (1954).
[19] Fox, J. J., N. Yung, J. Davoll, and G. B. Brown, *J. Am. Chem. Soc.*, **78**, 2117 (1956).
[20] Flaks, J. G., and S. S. Cohen, *J. Biol. Chem.*, **234**, 2981 (1959).
[21] Reichard, P., *Acta Chem. Scand.*, **9**, 1275 (1955).

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*THE AMINO ACID COMPOSITION AND C-TERMINAL SEQUENCE OF A CHEMICALLY EVOKED MUTANT OF TMV**

BY A. TSUGITA AND H. FRAENKEL-CONRAT

VIRUS LABORATORY, UNIVERSITY OF CALIFORNIA, BERKELEY

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Most naturally occurring strains of tobacco mosaic virus (TMV) have been found to show differences in their amino acid composition.[1, 2] When it was found that mutants could be artificially produced by means of chemical modification of the virus or its RNA,[3] the question arose whether such mutants would also show detectable changes in the composition or amino acid sequence of their protein. Because of recent advances in the methodology of amino acid analysis,[4] the detection of changes as small as the replacement of one amino acid residue per peptide chain has become possible. A preliminary survey of several different chemically evoked mutants suggests that such replacements actually occur. The detailed study of the amino acid composition of one particular mutant, to be reported here, indicates the replacement of one residue each of proline, aspartic acid, and threonine by leucine, alanine, and serine. Furthermore, it is shown that at least one of these changes takes place near the C-terminus of the peptide chain, thereby greatly affecting the susceptibility of the protein to attack by carboxypeptidase.

Experimental.—The RNA was isolated from TMV by the phenol method[5] with 10^{-3} *M* versenate as buffer.[6] The nitrous acid treatment was performed according to the method of Schuster and Schramm.[7] Other reactions used to produce mutants were with small amounts of dimethyl sulfate, and with *N*-bromosuccinimide, both at 0° and pH 6–8; details of these modifications will be reported elsewhere.

After the chemical treatment, the RNA was isolated by repeated alcohol precipitations and reconstituted with native protein by the standard procedure.[8] The extent of inactivation was determined by assay on the Xanthi variety of *N. tabacum*. For the detection of mutants, the reconstituted virus was applied to *N. sylvestris* and Java Tobacco. In confirmation of Gierer and Mundry,[3] we observed frequent local lesions on these systematically-diseased test plants when nitrous acid had been employed but almost never with untreated, reconstituted virus. In addition, we obtained local lesions with somewhat lesser frequency after treatment with the methylating and brominating agents listed above but hardly ever after a variety of other chemical modifications.

The local lesions were cut out and serially transferred (2–4 times) in the same hosts until only local lesions without systemic infection resulted. Subsequent

TABLE 1

ANALYSIS OF NITROUS ACID MUTANT (171) AND TMV*

	Mutant 171			Tobacco Mosaic Virus (TMV)				
Amino acid	24-hr hydrol.	48-hr hydrol.	Nearest integer value	24-hr hydrol.	48-hr hydrol.	Nearest integer value	Ramachandran[12]	Wittmann and Braunitzer[13]
Asp	16.6	16.8	*17*	17.3	18.1	18	*19*	18
Thr	14.0	13.2	*15*†	15.0	14.4	16†	*17*	16
Ser	14.7	13.1	(16–) *17*†	14.2	12.8	(15–) 16†	*18*	16
Glu	15.5	15.4	16	15.4	15.5	16	*17*	16
Pro	7.0	6.8	*7*	8.1	7.9	8	8	8
Gly	6.0	6.0	6	6.0	6.0	6	6	6
Ala	14.8	14.8	*15*	14.0	14.0	14	*15*	14
Cys/2	0.5	0.3	1	0.6	0.4	1	1	1
Val	13.4	13.7	14	13.2	13.7	14	14	14
Ileu	8.0	8.6	9	7.8	8.5	9	*9*	*8*
Leu	12.6	12.7	*13*	11.7	11.9	12	*13*	12
Tyr	3.9	3.8	4	3.9	4.0	4	4	4
Phe	7.9	7.9	8	7.9	8.0	8	8	8
Lys	2.0	2.1	2	2.1	2.0	2	2	2
Arg	11.1	11.0	11	11.1	10.9	11	11	11
Try	..	..	(3)	..	..	(3)	(2)	3
Total			158			158	164	157

* Average of duplicate or triplicate results. Maximum error = ±0.1. Calculation is based on Kjeldahl nitrogen using Prot. N content = 16.2%. Calculated as number of residues per gram mole virus protein (i.e., = 18,000 g) as analyzed after 24 and 48 hr of hydrolysis (6 *N* HCl, 108° in high vacuum-sealed tubes). Values for 72 hr of hydrolysis of TMV were not listed since they differed significantly only in regard to serine and threonine. In italics are values which show differences from present analysis of common TMV.

† Values for threonine after extrapolation to 0-time of hydrolysis (see Fig. 1) were 14.8 and 15.5 for the mutant and TMV, respectively; for serine the corresponding values were 16.3 and 15.4, respectively.

transfer to *N. tabacum* produced a systemic disease which varied in severity for different isolates but was more often quite severe and necrotising on this host. The virus mutants were harvested from *N. tabacum* 3 weeks after inoculation, and isolated, as usual, by differential centrifugation. They were again tested on the various test plants to ascertain that they had not changed biologically.

Protein was prepared from the virus by the acetic acid method.[9] The protein (6–12 mg) was then hydrolyzed for 24, 48, and, if possible, 72 hr in 1 ml of 6 *N* twice redistilled HCl sealed under high vacuum. The acid was then evaporated; the residue was redissolved in water and again evaporated, and then redissolved in pH 3.2 buffer, freed from insoluble humin, and applied to the ion exchange column of the Spinco Amino Acid Analyzer.[4]

For the carboxypeptidase treatment of viral protein, commercial crystalline

carboxypeptidase (Worthington Biochem. Corp., #CO570) was treated with diisopropylphosphofluoridate by the procedure of Gladner *et al.*[10] The protein was treated with one two-hundredth of its weight of carboxypeptidase at room temperature for various time periods up to 47 hr. A second addition of enzyme was made to digests held longer than 24 hr. At the end of the digestion period, the samples were adjusted to pH 4.3, the insoluble protein was removed, and the supernatant brought to pH 2 and directly applied to the Amino Acid Analyzer. Control experiments with the enzyme alone showed no detectable amounts of liberated amino acids. TMV gave the expected threonine (0.9 equivalents), and a small amount of serine in one 24-hr digest.

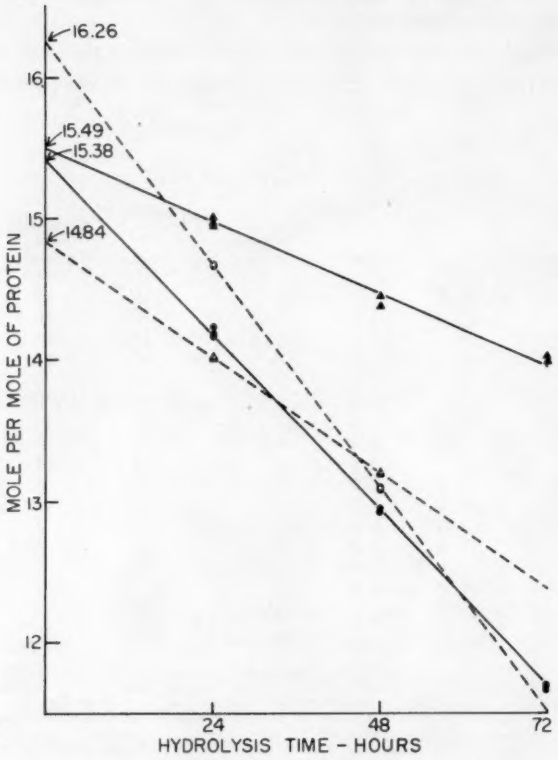

FIG. 1.—Decomposition of serine and threonine during acid hydrolysis. The results of individual analyses are plotted. Those for serine are indicated by circles, those of theonine by triangles. The open symbols are the results obtained with the mutant #171, the filled-in symbols those with common TMV. The intercept values obtained by extrapolation to zero time are given. (For experimental details, see text.)

Results and Discussion.—Several mutants obtained with nitrous acid or *N*-bromosuccinimide were analyzed in preliminary studies when the accuracy of analysis had not yet been stabilized. In each case one or several differences between the mutant and the starting material, common TMV, were detected. The strain which was selected for a thorough study was a nitrous acid mutant (#171) which had been obtained from an RNA preparation inactivated to about 99 per cent. Since mutants were observed with similar or greater frequency concomitant with much lesser inactivation, and, as stated above, only with certain inactivating agents and not with others, we conclude, in full accord with Mundry,[11] that mutagenesis and not selection is here operative. The mutant #171 gave only local lesions on *N. sylvestris* and Java Tobacco, and produced a mosaic disease on Turkish which was milder than that given by common TMV.

The results of the amino acid analysis of the mutant strain #171 and of TMV are listed on Table 1. Comparison of these analyses shows clearly a decrease by one residue in the number of aspartic acids, threonines, and prolines and an increase by one alanine, serine, and leucine residue. Also included in Table 1 is a comparison of the present analysis of common TMV with that reported by

Ramachandran,[12] and that derived from a summation of the 12 component peptides by Wittmann and Braunitzer.[13] It appears that the present analysis differs from Ramachandran's by 2 residues of serine, and one each of aspartic and glutamic acids, threonine, alanine, and leucine. With the exception of one isoleucine residue, the present analysis agrees completely with that obtained more laboriously by Wittman and Braunitzer.[13] Since isoleucine is liberated only slowly from peptide linkage, and since Wittman has recently also obtained values approaching our preferred value of 9 residues,[14] it would seem that this is more probably the correct value. The over-all outcome of this comparison of results obtained in different laboratories is astonishingly concordant. It thus appears that the technique of amino acid analysis has now advanced to a point where, under favorable circumstances, complete analysis of a protein containing 158 residues is possible with sufficient accuracy to establish the number of residues of almost all amino acids. One remaining slight uncertainty is the exact number of serine and threonine residues, which is due to the progressive decomposition of these amino acids during hydrolysis. Extrapolation of the resultant decay curves to zero time is the accepted procedure (see Fig. 1), but such extrapolation magnifies the observed error of each analysis and thus leads to a range of about one residue out of 16 between the highest and lowest extrapolated value. Thus, the listed serine and threonine content of TMV as well as of the mutant may possibly be in error by one residue.

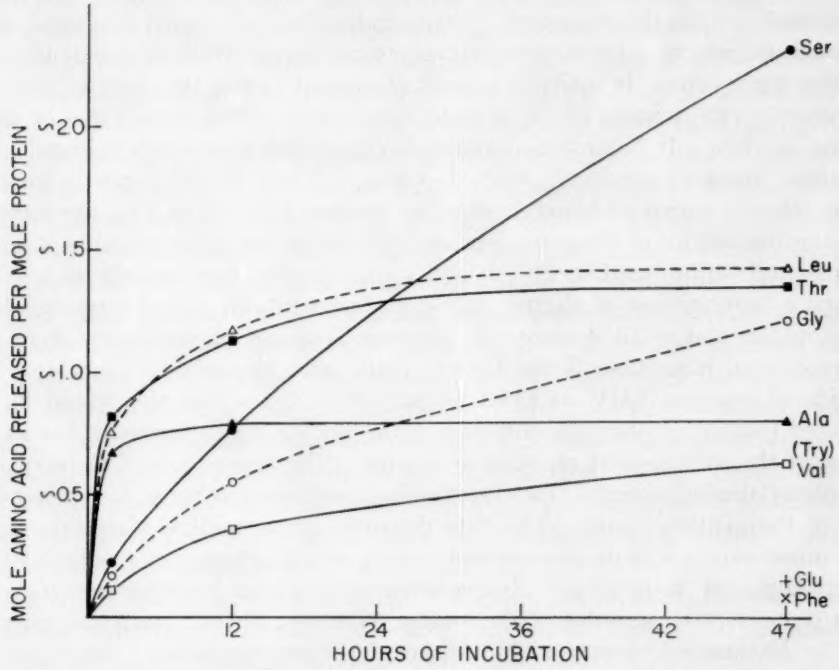

FIG. 2.—Carboxypeptidase digestion of the protein of mutant #171. The released amino acids were determined by means of the Amino Acid Analyzer after removal of the protein by isoelectric precipitation. The aliquot size after the longest (47-hr) enzyme treatment is ±10%, an error which could affect the absolute level of each amino acid, but not the relative amounts. (For experimental details, see text.)

The values reported by Wittmann[14] show a slightly greater range of uncertainty in this regard. The analysis and summation of the isolated peptides, each containing maximally only 6 serine residues, should be expected to give reliable values were it not for the fact that no details concerning such factors as hydrolysis times, extrapolation slope, and number of replicate analyses have as yet been reported for the isolated peptides.[13] The fact remains that the reported exchange of one threonine for a serine residue in mutant 171 is established with slightly less certainty than the other two amino acid replacements.

Treatment of TMV and all its natural strains with carboxypeptidase has been shown to lead to the release of one residue of threonine and no other amino acids from each peptide chain.[15, 16] This has been attributed to the since-established C-terminal -pro-ala-thr sequence[17, 18] since proline has been found in several other instances to present a block to carboxypeptidase action which may extend to its neighboring residue. It therefore seemed of interest to test the effect of carboxypeptidase on the protein of the mutant strain #171, which lacked one of the 8 proline residues. It became immediately evident that this strain behaved quite differently from all previously studied strains of TMV in this regard. Analysis of the released amino acids indicated, after as short a period as 2 hr, the presence of nearly one residue of threonine, leucine, and alanine and lesser amounts of serine, glycine, and valine (see Fig. 2). Analysis after varying time periods up to 47 hr showed a faster release of glycine, valine, and particularly several serine residues. Tryptophane and small amounts of glutamic acid and phenylalanine were also detected. An inspection of the known amino acid sequence of the C-terminal peptide of common TMV, as listed below,[19, 20] indicates that this would be the expected pattern of amino acid release if the proline which normally blocks the action of the enzyme were replaced by leucine. The appearance of glutamic acid and phenylalanine indicates that digestion has progressed as far as 15 residues into part of the peptide chains. The data definitely prove marked alteration of the C-terminal sequence in the mutant under study and the absence of proline from the degraded part of the molecule. Its replacement by a leucine residue represents the most probable interpretation of these data. The rest of the C-terminal sequence may be the same as in common TMV, although the presence of many serine residues in this part of the molecule renders the interpretation somewhat equivocal. Thus, according to an alternate interpretation of the relative amino acid release rates, the threonine in sixth position might be exchanged by a serine residue:

-ser-ser-phe-glu-ser-ser-ser-gly-leu-val-try-thr-ser-gly-*pro*-ala-thr-OH →
-ser-ser-phe-glu-ser-ser-ser-gly-leu-val-try-thr-ser-gly-*leu*-ala-thr-OH.

Further studies on the structure of this mutant are in progress.

The results reported clearly demonstrate that a mutant strain of TMV has been isolated which differs markedly from all previously studied mutants. It is impossible to prove unequivocally that this mutant was actually produced by the chemical treatment and did not originate spontaneously. However, the proportion of local lesions evoked on *N. sylvestris* by modified virus to those given by common TMV is certainly at least 100 to 1, and thus the probability greatly favors chemical origin for this mutant. As stated, other similarly isolated chemically induced mutants have also given indications of changes in amino acid composition.

In contrast, it has recently been reported by Wittmann[14] (a paper that became accessible to us only after completion of this study) that a nitrous acid mutant isolated by him has not shown any difference in composition from TMV. This important and well-documented finding, owing to its negative nature, is intrinsically neither definite nor final. While differences are, with luck, easily detectable, the absence of difference can be demonstrated only by exhaustive analysis. Thus the possibility that two amino acids are interchanged in position within one peptide segment can be excluded only by complete sequential analysis of each peptide. Furthermore, the problems connected with serine and threonine destruction, which were discussed above, leave some doubt as to the certainty of both the differences, and the absence of differences, in regard to these amino acids. However, it is quite possible that Wittmann's cautious and tentative conclusion will prove correct, and that he has studied a nitrous acid mutant of TMV which does not differ in its amino acid composition from the parent strain. If the complete amino acid sequence of the two were identical, this would lead one to the conclusion, which appears in no way unexpected, that certain parts of the polynucleotide chain carry the code for the amino acid sequence of the viral coat protein while other parts of the RNA chain are not concerned with the structure of this protein. It is of interest to note, in this connection, that Wittmann has studied a mutant which differs less markedly from the common strain than does the one studied by us. It has been reported[21] that so-called symptom mutants differing only in the severity of disease symptoms which they produce on *N. tabacum*, or in lesion size as noted on *N. glutinosa*, occur with very high frequencies of 50 per cent or more, quite in contrast to the much rarer differential host mutants on which we have concentrated our efforts. Thus it appears possible that the latter are more likely to show pronounced protein structural changes than the former. Such questions will become resolved only when statistically significant numbers of the various types of mutants produced by different chemical agents have been analyzed with regard to protein structure and other properties.

Summary.—The protein of a differential host mutant isolated after nitrous acid treatment and reconstitution of TMV-RNA differs from that of the parent strain in that 3 amino acid residues are replaced by three others (proline, aspartic acid, and threonine, by leucine, alanine, and serine). The composition of the parent strain appears identical with that of the *Vulgare* strain studied in Tübingen.

The action of carboxypeptidase on the mutant leads to extensive digestion, indicating that one of the changes, namely the replacement of proline by leucine, has occurred near the C-terminus of the peptide chain.

The implication of the fact that some chemically produced mutants differ in their protein composition, while others may not do so, as reported by Wittmann recently, appears of considerable interest in connection with the mechanism of coding of genetic properties by the RNA.

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[1] Knight, C. A., *J. Biol. Chem.*, **171**, 297 (1947).

[2] Aach, H. G., *Z. Naturforsch.*, **13b**, 426 (1958).

[3] Gierer, A., aud K. W. Mundry, *Nature*, **182**, 1457 (1958).

[4] Spackmann, B. H., W. H. Stein, and S. Moore, *Anal. Chem.*, **30**, 1190 (1958).

[5] Gierer, A., and G. Schramm, *Z. Naturforsch.*, **11b**, 138 (1956).
[6] Haschemeyer, R., B. Singer, and H. Fraenkel-Conrat, these PROCEEDINGS, **45**, 313 (1959).
[7] Schuster, H., and G. Schramm, *Z. Naturforsch.*, **13b**, 697 (1958).
[8] Fraenkel-Conrat, H., and B. Singer, *Biochim. Biophys. Acta*, **33**, 359 (1959).
[9] Fraenkel-Conrat, H., *Virology*, **4**, 1 (1957).
[10] Gladner, J. A., and H. Neurath, *J. Biol. Chem.*, **205**, 345 (1953).
[11] Mundry, K. W., *Virology*, **9**, 722 (1959).
[12] Ramachandran, L. K., *Virology*, **5**, 244 (1958).
[13] Wittmann, H. G., and G. Braunitzer, *Virology*, **9**, 726 (1959).
[14] Wittmann, H. G., *Z. Vererbungslehre*, **90**, 463 (1959).
[15] Harris, J. I., and C. A. Knight, *Nature*, **170**, 613 (1952).
[16] Knight, C. A., *J. Biol. Chem.*, **214**, 231 (1955).
[17] Niu, C. I., and H. Fraenkel-Conrat, *Biochim. Biophys. Acta*, **16**, 597 (1955).
[18] Niu, C. I., and H. Fraenkel-Conrat, *Arch. Biochem. and Biophys.*, **59**, 538 (1955).
[19] Gish, D. T. *Biochem. and Biophys. Res. Com.*, **1**, 67 (1959).
[20] We are indebted to Dr. D. T. Gish for permitting us to include this sequence, parts of which have not yet been published.
[21] Siegel, A., (private communication).

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*NON-VIABILITY OF STALK CELLS IN DICTYOSTELIUM**

BY WILLIAM F. WHITTINGHAM AND KENNETH B. RAPER

DEPARTMENTS OF BOTANY AND BACTERIOLOGY, UNIVERSITY OF WISCONSIN

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The Dictyosteliaceae, a family of simple amoeboid slime molds, are distinguished by their capacity to construct multicellular fruiting structures through the aggregation and orderly differentiation of large numbers of previously independent myxamoebae. The onset of the fruiting stage is marked by a dramatic shift from a free-living state to an interdependent population in which the cells become conspicuously elongated and orient themselves toward inflowing streams of myxamoebae which they join to form a cell community, or pseudoplasmodium. Subsequent development is characterized by a progressive integration of the assembled cells, the mass of myxamoebae behaving as a coordinated functioning whole wherein the differentiation of each member cell is influenced, apparently, by the remainder of the cell aggregate. The end result is the construction of a well-proportioned fructification, or sorocarp, consisting of two types of contrasting and strongly differentiated cells: spores, or propagative cells, that form an apical rounded spore head, or sorus; and polygonal, vacuolated, pith-like cells that comprise the stalk, or sorophore, which supports the sorus (or sori in branched forms).

It is this relatively simple behavioral pattern involving progressive specialization and ultimate differentiation into two cell types that has led investigators to utilize the Acrasieae, and particularly species of *Dictyostelium*, for researches on cell differentiation. Most students of these slime molds have believed the two cell types to be not only morphologically but also functionally distinguishable. Harper's statement of this concept expresses the generally accepted view: "The very cells which initiate and carry out the process of building the sorophore are sacrificed in the interests of giving the remainder a better place in the sun";[1] i.e., a substantial

proportion (often approximately 25%) of the cells within a fruiting population becomes incapable of propagating the species. This notion was first questioned by Sussman in 1951[2] in a paper wherein he described a method for clonal plating and isolation of these slime molds. Plating mixed populations of different cell types, he calculated from indirect evidence that in *Dictyostelium purpureum* and in *D. mucoroides* the probability of stalk cells giving rise to myxamoebae was the same as for spores, if both kinds of cells were placed in a similar environment. The same conclusion has been reaffirmed in two subsequent papers.[3, 4] Thus he implies that the difference between the stalk cells and spores is primarily a morphological rather than a functional discontinuity. If this were true, much of the published research relating to these organisms would be of questionable significance, for many investigators have emphasized their usefulness as relatively simple models of systems of cellular differentiation that may be operative in higher organisms.

As a result of observations extending over a period of many years which included, more particularly, the detailed study of sorocarp formation in *Dictyostelium discoideum* by Raper and Fennell,[5] we have long questioned Sussman's contention that "all of the cells, regardless of state of development or position in the sorocarp, are identical in their capacity to originate clonal growth with subsequent formation of normal fruiting structures."[2] Only recently have we had an opportunity to undertake a careful investigation of this matter. We have worked specifically with *Dictyostelium purpureum*, strain #3645, and the data presented herewith relate to this culture. However, from comparative study of the three cultures investigated by Sussman, which were received from one of us (K. B. R.), we believe that our results are as meaningful for them and for other members of the Dictyosteliaceae as they are for the strain upon which our attention has been concentrated. Small fragments of stalks and isolated stalk cells of *Dictyostelium purpureum* were transplanted and examined in environments which should have permitted establishment of myxamoeboid clones if stalk cells were capable of regrowth. Such revegetation in the proved absence of spores, encysted myxamoebae (microcysts),[6] or vegetative cells has not been observed.

A water suspension of slime mold spores and *Escherichia coli* was streaked on an agar medium containing 0.1 per cent lactose and 0.1 per cent peptone (designated 0.1 *L-P*) and the plates incubated at room temperature (22°C) until the characteristic wheel-like aggregations of myxamoebae appeared along the line of inoculation. Small agar blocks containing the largest aggregates were then transferred to a water-agar substrate and further incubated in one-sided light to allow extended migration of the pseudoplasmodia in the absence of vegetative myxamoebae, spores, and microcysts. In other cases sorocarps were allowed to develop *in situ* on 0.1 *L-P* agar plates. Under each of these conditions, the stalks developed horizontally and in contact with the substrate surface, as is illustrated in Figures 1 and 2. Examination with a 4 mm objective (440 ×) permitted us to select portions of stalks that exhibited the least surface irregularity. Previous experience had shown that rounded cellular bodies (micryocysts?) not having the characteristic appearance of most stalk cells or of irregularly-vacuolated cells external to the sorophore sheath, often adhered to the stalk surface (Fig. 8). Portions of stalk not exhibiting either of these types of peripheral cells served as the inoculum source. Fragments containing 15 to 250 cells (Fig. 7) were dissected from stalks

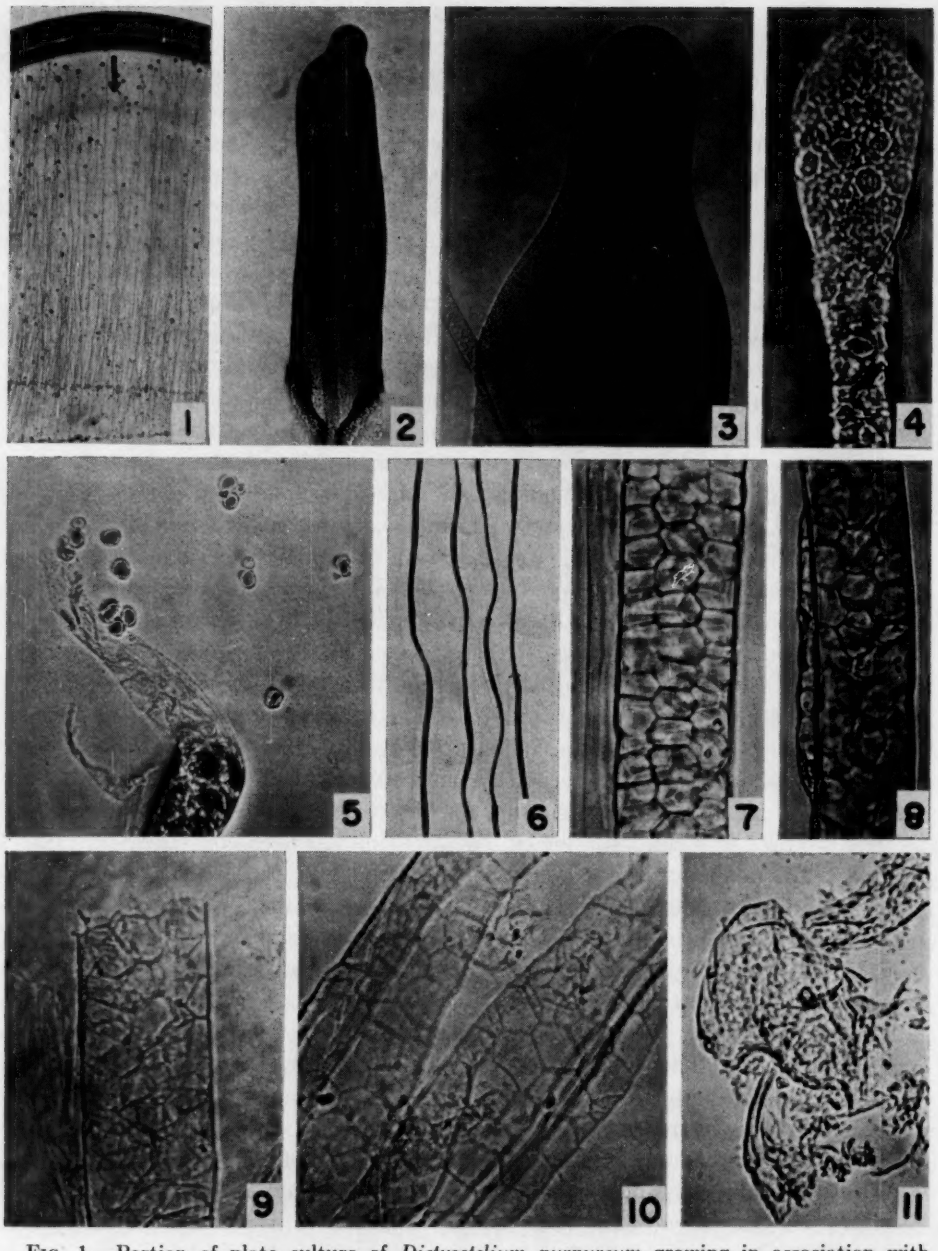

FIG. 1.—Portion of plate culture of *Dictyostelium purpureum* growing in association with *Escherichia coli* on 0.1 L-P agar, incubated at 22°C with sorocarps strongly oriented toward light source, indicated by arrow. FIG. 2.—Sorogen in contact with agar and moving in direction of light source; note that the stalk extends into the apical tip. × 55. FIG. 3.—Apical area of similar sorogen slightly compressed by cover glass to reveal expanded, funnel-shaped terminus where differentiation of stalk-forming myxamoebae occurs. × 110. FIG. 4.—Terminal portion of developing sorophore removed from sorogen showing completely-differentiated cells within rigid sorophore sheath below, and cells in process of vacuolation in the still-elastic sheath above. × 425. FIG. 5.—Portion of a stalk terminus similar to the preceding, but with incompletely-differentiated cells expressed from the enveloping sheath and dispersed on agar surface preparatory to isolation. × 235. FIG. 6.—Four mature stalks of the type from which fragments free of extraneous cells were removed for tests. × 20. FIG. 7.—Section of a mature sorophore showing the characteristic parenchyma-like cells that comprise the central cylinder. × 550. FIG. 8.—Section of a similar sorophore with a group of cells lying outside the sorophore sheath and differentiated, in this case, in the manner of stalk cells. × 550. FIGS. 9 and 10.—Mature sorophores broken in transverse and longitudinal directions, respectively; note that the sorophores break independently of cell boundaries and that the constituent cells do not separate from each other. × 550. FIG. 11.—Fragment of a mature sorophore following mild homogenization; no trace of its earlier cellular structure remains. × 550.

not older than two days and transferred on small blocks of agar (to prevent desiccation) to either 0.1 *L-P* or Sussman's *SM* medium.[2] Each fragment was again examined at 440 × to estimate the number of cells present. If no spores or microcysts were observed, one drop of a bacterial suspension (*E. coli* on 0.1 *L-P*; *Aerobacter aerogenes* on *SM* agar) was placed on the fragment to provide nutrient for any emergent myxamoebae. A third examination confirmed that the fragment remained within the confines of the added bacterial inoculum. Spores planted in a similar manner served as controls. The plates were incubated at 22°C for 6 days and examined daily for the presence of myxamoebae and/or fruiting bodies. In all experiments, myxamoebae appeared on the control plates within two days and normal sorocarps developed by the third day.

The results of 6 experiments recorded in Table 1 show that only 1 stalk fragment out of 81 initiated a clone of myxamoebae. The experiments utilizing *SM* medium simulated the cultural environment employed by Sussman,[2] whereas 0.1 *L-P* agar has been much used in our laboratory. Rapid growth and normal development of *D. purpureum* occurs on each substrate in association with *E. coli* or *Aerobacter aerogenes*. It does not seem unreasonable to attribute the single exception to chance contamination by a myxamoeba, microcyst, or spore that was overlooked during the microscopic examinations.

TABLE 1

RESULTS OF PRELIMINARY EXPERIMENTS TO TEST THE ABILITY OF STALK CELLS OF *Dictyostelium purpureum* TO INITIATE CLONES OF MYXAMOEBAE

Medium	Number of stalk fragments tested	Size of fragments (Number of cells)	Number of fragments initiating vegetative clones
0.1 L-P	9	150–250	0
0.1 L-P	12	150–250	1
0.1 L-P	24	15–70	0
SM	12	150–250	0
SM	12	150–250	0
SM	12	15–70	0

Sussman had used dissociated cells in his tests, whereas we had transplanted stalk fragments without disrupting intercellular relationships. Recognizing that the behavior of individual cells, including any potential regenerative capacity, might be different under these contrasting conditions, various techniques were investigated in attempts to prepare suspensions in which the stalk cells were dispersed as separate entities. Selected stem fragments were placed in small amounts of physiological salt solution in test tubes and vigorously triturated against the side of the vessel (as recommended by Sussman),[2] but no single, intact cells were dislodged from them, albeit the fragments were conspicuously broken and distorted. (Examples of stalks broken transversely and longitudinally are seen in Figs. 9 and 10). Similar fragments were then placed in small conical tissue homogenizers with differing amounts of salt solution and, employing varying degrees of pressure, efforts were made to disrupt the stalks sufficiently to release single cells. The objective was not attained. Rather the whole structures were dismembered to the point where virtually all evidence of their prior cellular structure was obliterated (Fig. 11), a condition seen earlier in *D. discoideum*[5] when sorophores were crushed. In no case did we succeed in separating cells from portions of a sorophore wherein such cells had become sufficiently differentiated to assume angular outlines incident to final vacuolation and compression within the enveloping cellulose sheath

that surrounded them (see Raper and Fennell,[5] for details of sorophore formation and structure). In no case did we observe growth of *Dictyostelium* when such homogenates were added to suitable nutritive bacteria on favorable culture media.

Other methods of freeing the differentiated stalk cells under conditions that might favor any possible regenerative capacity were considered. Implantation of stalk fragments in the presence of cellulose-decomposing bacteria offered an attractive possibility. Earlier studies had demonstrated that the myxamoebae of *Dictyostelium discoideum* would feed and vegetate upon the cells of *Cellulomonas fima* and that, conversely, these bacteria were capable of decomposing the cellulosic component of mature sorophores.[5,7] It was reasoned, therefore, that the same relationships might apply to *D. purpureum*, and that, in the course of these counteractivities, *Cellulomonas* might, in degrading the cellulose, release protoplasts (if such still persisted but for which there was no visual or cytological evidence) which then being freed could phagocytize the bacterial cells. No growth of the slime mold occurred in any case where a carefully selected stalk fragment was so implanted, although satisfactory growth and fructification did occur within three days in control plates where either spores or myxamoebae were introduced into colonies of *Cellulomonas* under like circumstances. As in the earlier experiments, the age of the stalk fragments tested were from one to two days.

Implantation of stalk fragments on non-nutrient agar in the presence of a pectin-destroying bacterium, *Erwinia carotovora* (which it was thought might degrade the intercellular cementing substance if perchance this represented pectin) yielded similar negative results after prolonged incubation. Controls seeded with spores, but otherwise duplicating the tests, yielded mature sorocarps within three days.

Like Harper,[2] and Brefeld[8] before him, the authors concluded that fully differentiated stalk cells of *Dictyostelium* are indeed sacrificed in the construction of the fruiting body and are no longer capable of propagating these slime molds.

It now became of special interest to determine at what stage of the differentiative process cells destined to become elements of the stalk do in fact lose their regenerative capacity. It has long been known that undifferentiated myxamoebae are capable of reverting to the vegetative stage if separated from the fruiting organization of which they are an integral part and placed in the presence of nutritive bacteria, this applying equally well to the aggregative stage, the migrating phase, if such occurs, and at least the earlier stages of sorocrap formation.[9] The above experiments indicate with equal clarity that stages are reached, however, where a cell becomes irrevocably either an encapsulated spore, and so retains its reproductive ability, or a vacuolated stalk cell, and so loses this capacity. The latter phenomenon was investigated.

Dictyostelium purpureum builds long-stalked sorocarps in the direction of the light source when cultivated under favorable conditions in one-sided light, as seen in Figure 1. In developing sorocarps of this type with stalks up to 6–7 cm in length, the still-undifferentiated sorogens (Figs. 2, 3) within which sorophores are forming are often far removed from any free-living myxamoebae or microcysts. Such horizontally oriented sorophores afford ideal material for detecting the "point of no return" in the differentiation of stalk cells. When the sorogen is in contact with the agar, one can, by careful manipulation, pull the stalk from inside this, including the terminal funnel-shaped portion wherein the nascent stalk cells

are undergoing rapid and final vacuolation. Figure 3 illustrates such a sorogen with the site of active sorophore formation clearly evident. The apical portion of a sorophore removed from a comparable sorogen is shown in Figure 4, and some detail of its internal cellular composition is revealed. In the proximal portion cells are strongly vacuolated, compressed, and cemented together, forming a rigid structure, whereas at the still-expanded distal end some uncompacted cells appear as rounded, vacuolate myxamoebae within a field of less-differentiated cells.

In the first series of experiments, thirteen sorophores were removed from their sorogens in the manner described, carefully washed to free them of external amoeboid cells, placed on non-nutrient agar, and examined at 440 × to insure the absence of all extraneous cells. Short, intact apical segmets were then removed to other locations on the plate and covered with a heavy suspension of *E. coli* cells. No slime mold growth occurred in any case.

The total absence of growth led us to suspect that the uncemented cells had been washed free from the open end of the sorophore and that our test was, in reality, comparable to those previously described for intact segments. Individual cells from the formative area were then isolated and tested. Starting with a properly isolated and prepared sorophore, cells from the area of rapid vacuolation were expressed by the application of a slight lateral pressure, much as one might remove the ascospores from an ascus of *Neurospora*. These were then dispersed on the agar surface and individual cells removed and placed in environments suitable for revegetation if this capacity still remained.

Two experiments were performed. In the first of these, the portion of the sorophore from within the sorogen was carefully isolated. Attention was focused on the apical, funnel-shaped region where some of the cells already possessed conspicuous walls, being enlarged, strongly vacuolate, and rounded but not mutually compressed, i.e., differentiation was progressing rapidly but the cells had not yet assumed their final and definitive shapes (Fig. 4). Six single, strongly vacuolated, rounded, walled cells were isolated with fine glass needles and transferred to non-nutrient agar on small blocks of the same medium. A drop of a heavy suspension of *E. coli* was placed on each cell. No evidence of growth was observed within three weeks. In another case, a group of four or five unwalled cells handled in a similar manner exhibited growth with normal fruiting within three days.

In the second experiment a similar portion of a stalk was carefully isolated and the non-differentiated pre-spore myxamoebae surrounding it were removed by gently dragging it along the surface of the substrate. The stalk was then compressed to eject the rounded, partially differentiated cells from the stalk sheath, and these were dispersed as seen in Figure 5. Examination at 440 × confirmed these to be enlarged stalk cells, not yet mutually compressed, interspersed with a few non-differentiated cells. Forty-four of the rounded, vacuolate cells were isolated and incubated on non-nutrient agar with added *E. coli*. After five days, three spots exhibited mature sorocarps, whereas the remaining forty-one showed no slime mold growth after three weeks. It is presumed that the three isolations that yielded colonies of slime mold resulted from undetectably less-differentiated cells which still retained regenerative capacity, or that they shielded still undifferentiated myxamoebae which escaped our examination at the time of transfer.

We conclude, on the bases of the above tests, that fully differentiated stalk

cells lose completely their capacity to regenerate in the process of vacuolation and mutual compression leading to the formation of a mature parenchyma-like sorophore. Additionally, we are led to believe that the same occurs in any differentiating stalk cell once the central vacuole has expanded beyond the dimensions of the prior amoeboid cell body and a firm wall has been laid down by the aging protoplast. Obviously, there is a stage between this and that of an undifferentiated myxamoeba where cell vitality is gradually lost. The experiments performed to date do not permit us to state more precisely the cytological changes associated with this loss, although Bonner[10] has observed a progressive degeneration of the nucleus and we regularly observe active Brownian movement of particles within the vacuoles of fully differentiated stalk cells as shown in Figures 7 and 8. The state of irreversible function among the stalk cells of *Dictyostelium purpureum* thus appears to occur near the base of the funnel-like expansion in the developing sorophore and to be represented by those cells which show substantial vacuolation and enlargement, but prior to the time they become mutually compressed, hence form integral parts of the rigid stalk. Apparently, differentiation occurs fairly rapidly, for these changes occur within a relatively narrow zone and few cells of intermediate size can be distinguished.

How, then, can one account for the conflicting results reported and reaffirmed by Sussman and those obtained in the present investigation. Since all of his clonal platings that are germane to the possible regeneration of stalk cells were made with populations of mixed cell types, and his contention that stalk cells revegetate was based upon plaque formation by cells of unspecific origin, a number of possible explanations suggest themselves: (1) a substantial and unappreciated number of undifferentiated cells may have been carried over with the sorocarps that were triturated and examined, for the presence of myxamoebae in the suspensions was mentioned; (2) the entire sorocarps analyzed[11] may have been somewhat immature, hence contained in addition to spores and mature stalk cells a greater number of undifferentiated myxamoebae than was realized; (3) another possibility, believed highly improbable, is that the so-called stalk cells in some way stimulated the germination of spores, for the number of plaques did not exceed the number of spores present but rather the number of plaques anticipated upon the basis of plating efficiencies obtained with spores alone; (4) different strains of *Dictyostelium purpureum* were studied in the two investigations, but long and careful comparative observations of sorocarp formation and structure in each of these suggest no bases for dissimilar results; (5) the most probable explanation could be a misinterpretation by Sussman of the small, rounded, vacuolated cells with heavy walls which he called stalk cells. From his description, and from the methods he employed, the authors are led to conclude that there were few characteristic stalk cells in his suspensions, a view strongly supported by our inability to obtain single, dissociated stalk cells except from the narrow zone in the developing sorophore where these undergo rapid vacuolation. We believe the small, rounded cells seen in his preparations probably represented a cell type not usually associated with a functioning stalk cell. This could account for an apparently high viability of so-called stalk cells in Sussman's experiments but a negligible incidence of reversion, if any, in the present work.

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[1] Harper, R. A., "Morphogenesis in *Dictyostelium*," *Bull. Torrey Bot. Club*, **53**, 229–268 (1926).

[2] Sussman, Maurice, "The origin of cellular heterogeneity in the slime molds, Dictyosteliaceae," *J. Exp. Zool.*, **118**, 407–417 (1951).

[3] Sussman, M., "The biology of the cellular slime molds," *Ann. Rev. Microbiol.*, **10**, 21–50 (1956).

[4] Sussman, M., and Raquel R. Sussman, "Cellular interactions during the development of the cellular slime molds," in *Cellular Mechanism in Differentiation and Growth*, ed. Dorothea Rudnick (Princeton: Princeton Univ. Press, 1956), pp. 125–154.

[5] Raper, Kenneth B., and Dorothy I. Fennell, "Stalk formation in *Dictyostelium*," *Bull. Torrey Bot. Club*, **79**, 25–51 (1952).

[6] Blascovics, Joan C., and K. B. Raper, "Encystment stages of *Dictyostelium*," *Biol. Bull.*, 113, 58–88 (1957).

[7] Raper, Kenneth B., "Growth and development of *Dictyostelium discoideum* with different bacterial associates," *Jour. Agr. Res.*, **55**, 289–316 (1937).

[8] Brefeld, Oscar, "*Polysphondylium violaceum* und *Dictyostelium mucoroides* nebst Bemerkungen zur Systematik der Schleimpilze," *Untersuchungen aus dem Gesammtgebiet der Mykologie*, **6**, 1–34 (1884).

[9] Raper, Kenneth B., "Pseudoplasmodium formation and organization in *Dictyostelium discoideum*," *J. Elisha Mitchell Sci. Soc.*, **56**, 241–282 (1940).

[10] Bonner, John T., *The Cellular Slime Molds* (Princeton: Princeton Univ. Press, 1959).

[11] Sussman, M., *loc. cit.* (1951), with special reference to suspensions III and IV cited in Table 2, p. 414.

SIGNIFICANT STRUCTURES IN LIQUIDS, V. THERMODYNAMIC AND TRANSPORT PROPERTIES OF MOLTEN METALS

BY CHARLES M. CARLSON, HENRY EYRING, AND TAIKYUE REE

DEPARTMENT OF CHEMISTRY, UNIVERSITY OF UTAH

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Thermodynamic Properties.—Metals melt at comparatively low temperatures. This is because positive metal ions rather than atoms become randomly oriented in the melting process. Since the ions are only about a third as large as the atoms, the holes needed are only a third as large, and so ΔH is only a third as large. The effect of this may be seen by recalling the equation $\Delta H/T = \Delta S$. Since ΔH is only a third as large for ions while ΔS is not greatly different, the temperature of melting T must be correspondingly lower. This hypothesis that ions are important in metals is substantiated by viscosity data[1] which indicates that the hole size in metals is relatively small compared with the atom volume, although normal as compared with the ion volume.

Any hole theory for liquid metals should incorporate this idea. Kincaid and Eyring[2] applied Hirschfelder and Eyring's theory of liquids[3] to liquid mercury to predict many of its thermodynamic properties. White[4] used the partition function of Walter and Eyring[5] to calculate the thermodynamic properties of several molten metals. He calculated the viscosities of some of them as well.

The first part of this paper contains thermodynamic properties calculated for molten metals by the method of significant structures of Eyring, Ree, and Hirai.[6] The second portion discusses the application of the method of significant structures

in predicting the viscosities and self-diffusion coefficients of molten metals at various temperatures and pressures.

Application of the method of significant structures to metals gives the molar partition function

$$f = \left\{\frac{e^{E_s/RT}}{(1 - e^{-\theta/T})^3}\,[1 + n_h \cdot e^{-aE_s/n_hRT}]\right\}^{\frac{V_s}{V}N} \left\{\left(\frac{f_1 eV}{N}\right)^2 + \frac{2f_2 eV}{N}\right\}^{\left(1 - \frac{V_s}{V}\right)N} \quad (1)$$

where

$$f_1 = \frac{g(2\pi m_1 kT)^{3/2}}{h^3} \quad (2)$$

$$f_2 = \frac{(2\pi m_2 kT)^{3/2}}{h^3} \frac{8\pi^2 IkT}{2h^2} \frac{(e^{D/kT} - 1)}{(1 - e^{-h\nu/kT})} \quad (3)$$

and

$$n_h = n\left(\frac{V}{V_s} - 1\right). \quad (4)$$

The different symbols are defined as follows: E_s, θ, and V_s are the potential energy, the Einstein temperature, and the solid molar volume at the melting point; V is the molar volume of the liquid; n and a are parameters to be fitted to the experimental liquid data; m_1, m_2, D, I, and ν are the masses of the monatomic and diatomic metal molecules, the dissociation energy, the moment of inertia, and ground state vibrational frequency of the diatomic molecule, respectively; g, T, R, N, k, and h are the electronic degeneracy factor of the metal atom, the absolute temperature, the gas constant, Avogadro's number, Boltzmann's constant, and Planck's constant, respectively.

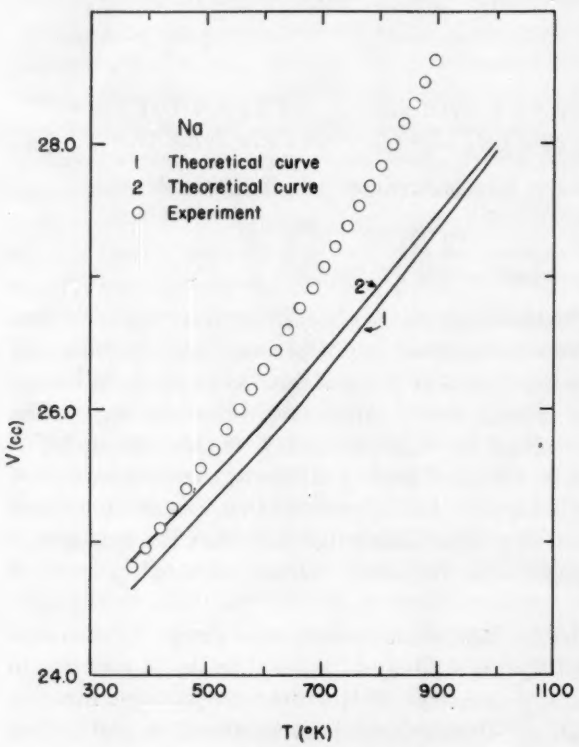

FIG. 1—Volume versus temperature for sodium. Curves 1 and 2 were calculated using values of $n = 25$, $a = 0.002500$ and $n = 30$, $a = 0.006000$, respectively. The experimental points are from ref. 8.

An Einstein solid is assumed. Monatomic and diatomic terms are included in the gas-like part of the partition function to account for the coexistence of monatomic and diatomic species. The factor $(e^{D/RT} - 1)$ terminates the oscillator partition function at the dissociation energy, D.

TABLE 1

PARAMETERS USED IN THE CALCULATIONS

	Na	Hg	Cu	Pb
V_s (cc)	24.17[8]	14.13[9]	7.641[10]	18.80[11]
E_s (kcal/mole)	25.75[8]	15.40[12]	79.80[13]	46.72[14]
θ (°K)	93.75[8]	60[12]	174[13]	56.25[14]
I (gm cm^2 × 10^{38})	1.8100[15]	(18.137)[16]	(2.5080)[17]	(10.752)*
$w = \nu/c$ (cm^{-1})	159.23[15]	(36)[15]	266.1[18]	159.22[15]
D_o (kcal/mole)	18.2[8]	(3.5)[19]	46.0[18, 20]	(23.00)[15]

* Interatomic radius used for calculating I was estimated from interatomic radii of similar molecules.

TABLE 2

MELTING POINT PROPERTIES OF METALS

	n	a		T_M (°K)	V_M (cc)	ΔS_M (eu)
Na	25	0.002500	Calc	371.01	24.64	0.94
			Obs	371.01	24.81[8]	1.68[8]
Na	30	0.006000	Calc	371.01	24.85	1.39
			Obs	371.01	24.81[8]	1.68[8]
Hg	45	0.01500	Calc	234.29	14.55	1.99
			Obs	234.29	14.65[9]	2.34[21]
Cu	35	0.02300	Calc	1,356.2	8.00	2.19
			Obs	1,356.2	7.96[10]	2.29[21]
Pb	45	0.009000	Calc	600.6	19.25	1.58
			Obs	600.6	19.42[11]	1.93[14]

TABLE 3

BOILING POINT PROPERTIES OF METALS

	n	a		T_B (°K)	V_B (cc)	ΔS_B (eu)	Q (%)
Na	25	0.002500	Calc	1,192	29.08	17.04	13
			Obs	1,156.1[8]	(30)	17.84[8]	17[8]
Na	30	0.006000	Calc	1,207	29.17	16.86	12
			Obs	1,156.1[8]	(30)	17.84[8]	17[8]
Hg	45	0.01500	Calc	648	16.50	20.39	0
			Obs	630[21]	15.75[21]	22.43[21]	0
Cu	35	0.02300	Calc	2,906	8.75	24.0	2.4
			Obs	2,855[21]		25.4[21]	
Pb	45	0.009000	Calc	2,056	21.60	19.77	0.88
			Obs	2,024[14]		21.19[14]	

TABLE 4

CRITICAL CONSTANTS OF METALS

	n	T_c(°K)	P_c (Atm)	V_c (cc)
Na	11	4,230	1,163	96.6
Na	6	3,100	729.3	101.0
Hg	11	1,552	610	63.7
Cu	11	10,033	8,681	30.6
Cu	6	8,198	6,465	31.4
Pb	11	8,390	3,500	68.2
Pb	6	6,266	2,530	68.3

TABLE 5

VISCOSITY OF LIQUID SODIUM*

T(°K)	η_{obs} (cp)[8]	η_1 (cp)	η_2 (cp)	η_3 (cp)
371.01	0.690	0.691	0.690	0.690
400.0	0.599	0.575	0.568	0.578
500.0	0.413	0.391	0.393	0.385
600.0	0.321	0.310	0.321	0.302
700.0	0.266	0.262	0.275	0.253
800.0	0.231	0.230	0.244	0.219
900.0	0.207	0.208	0.223	0.193
1,000.0	0.189	0.191	0.208	†
1,100.0	0.176	0.178	0.193	...
1,200.0	0.165	0.166	0.183	...

* For η_1, $b = 0$ and $r = 5.400$; for η_2, $b = 0.006906$ and $r = 4.936$; and for η_3 the activation energy is zero and $r = 3.942$.

† Experimental volumes above 900°K unavailable for calculation.

TABLE 6

SELF-DIFFUSION COEFFICIENT OF LIQUID SODIUM

(D's in units of cm²/sec × 10^{-5})

T (°K)	D_{obs}[26]	D_1 (ξ = 5.40)	D_2 (ξ = 5.40)	D_3 (ξ = 5.44)	D_4 (ξ = 5.19)
371.01	4.07	4.01	4.01	3.99	4.18
400.0	5.17	5.20	5.26	5.14	5.20
430.0	6.40	6.40	6.36	6.43	6.35
460.0	7.70	7.73	7.73	7.76	7.60
500.0	9.52	9.56	9.50	9.64	9.42

TABLE 7

VISCOSITY OF LIQUID MERCURY*

T (°K)	η_{obs} (cp)[22]	η_1 (cp)	η_2 (cp)
250	1.884	1.820	1.884
300	1.516	1.539	1.488
350	1.308	1.342	1.308
400	1.181	1.203	1.180
450	1.088	1.099	1.108
500	1.021	1.007	1.045
550	0.971	0.926	0.996
600	0.931	0.852	0.959

* For η_1, $b = 0$ and $r = 2.070$; for η_2, $bE_s/R = 4.999$ and $r = 2.809$.

TABLE 8

SELF-DIFFUSION COEFFICIENT OF LIQUID MERCURY

(D's in units of cm²/sec × 10^{-5})

T (°K)	D_{obs}[27]	D_1 (ξ = 6.20)	D_2 (ξ = 6.27)	D_3 (ξ = 6.24)
250	1.12	1.07	1.02	1.03
300	1.57	1.52	1.55	1.53
350	2.00	2.03	2.06	2.07
400	2.40	2.59	2.61	2.62

TABLE 9

PRESSURE VARIATION OF V_s FOR MERCURY

P (kg/cm²)	V_s (cc)
1	14.13
2,000	14.06
4,000	13.99
6,000	13.93
8,000	13.88
10,000	13.83
12,000	13.79

TABLE 10

PRESSURE VARIATION OF VISCOSITY OF MERCURY AT 303.16°K

P (kg/cm²)	η_{obs} (cp)	η_1 (cp)	η_2 (cp)
1	1.516	1.507	1.476
2,000	1.585	1.581	1.514
4,000	1.663	1.674	1.574
6,000	1.742	1.794	1.648
8,000	1.822	1.863	1.723
10,000	1.912	1.903	1.836
12,000	2.007	2.005	1.979

TABLE 11

PRESSURE VARIATION OF VISCOSITY OF MERCURY AT 348.16°K

P (kg/cm²)	η_{obs} (cp)	η_1 (cp)	η_2 (cp)
1	1.340	1.351	1.302
2,000	1.400	1.437	1.332
4,000	1.463	1.523	1.388
6,000	1.528	1.603	1.434
8,000	1.598	1.703	1.548
10,000	1.674	1.759	1.622
12,000	1.759	1.827	1.699

For all of the metals studied E_s and θ were determined in the same manner as for NaBr and KBr in paper III of this series.[7] That is, the two experimental quantities, energy of sublimation and vapor pressure, were used to fit the solid partition function at the melting point. The calculation of the liquid properties followed the procedure of Eyring, Ree, and Hirai.[6]

TABLE 12

PRESSURE VARIATION OF SELF-DIFFUSION COEFFICIENT FOR MERCURY AT 303.16°K

(D's in units of cm²/sec × 10^{-5})

P (kg/cm²)	D_{obs}[27]	D_1	D_2	D_3
1	1.63	1.56	1.58	1.55
2,000	1.56	1.49	1.54	1.48
4,000	1.48	1.41	1.49	1.41
6,000	1.43	1.32	1.42	1.35
8,000	1.36	1.27	1.36	1.29

Calculations have been carried out for sodium, mercury, copper, and lead. The melting point, the boiling point properties, and the critical constants have been calculated for these metals. For sodium and mercury the molar volume has been plotted as a function of temperature, and for mercury two isotherms have been plotted.

The parameters used in the calculations and their sources are summarized in Table 1. Parentheses enclose uncertain values. These uncertainties have but slight ffeect on the values of n and a.

Tables 2, 3, and 4 give the various properties calculated. The calculated melting temperatures agree exactly with observed values since they were used in determining n and a.

T_M, V_M, and ΔS_M are the melting temperature, liquid volume per mole at the melting point, and entropy of fusion; and the subscripts B and C indicate boiling and critical properties, respectively. Q is the mole per cent of diatomic species in the vapor at the boiling point. As can be noted either of two sets of the parameters, n and a, work well for sodium. Alternative values of n with $a = 0$, since a is without effect near the critical point, were used to determine the critical constants. In general, n must be given values

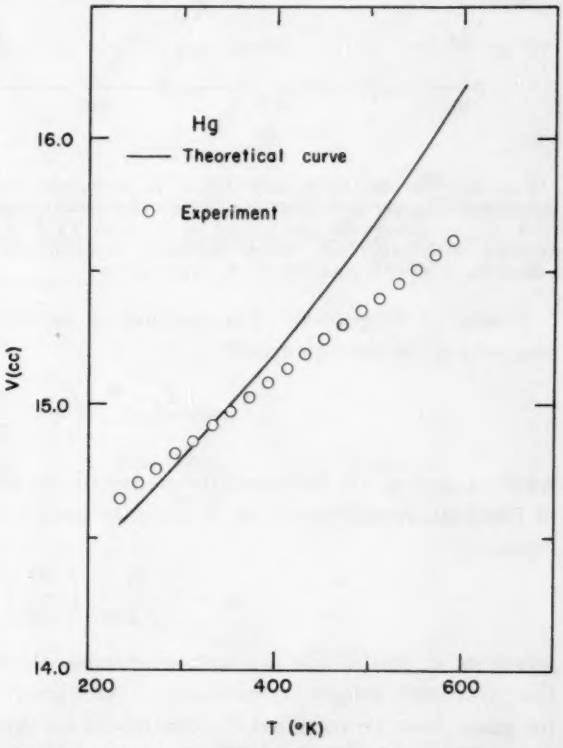

FIG. 2.—Volume versus temperature for mercury. The theoretical curve was calculated using $n = 45$, $a = 0.01500$. The experimental points were taken from ref. 22.

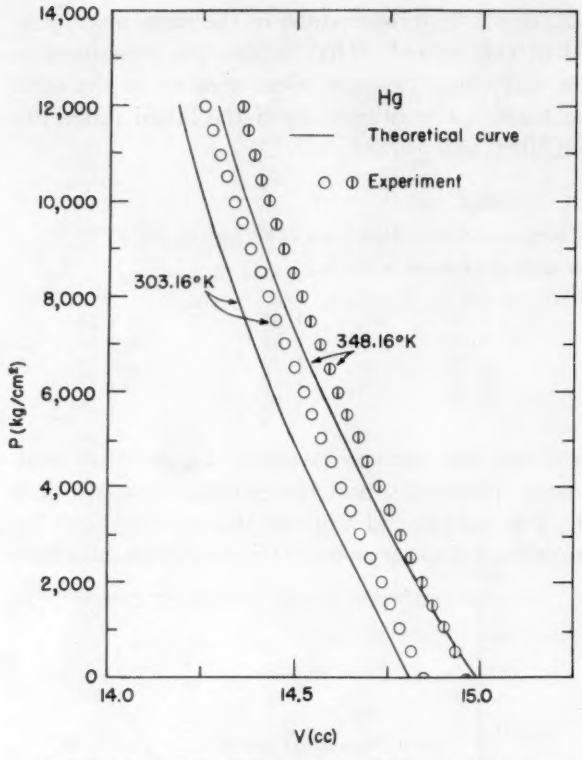

FIG. 3.—The 303.16°K and 348.16°K isotherms for mercury. The theoretical curves were calculated using $n = 45$, $a = 0.01500$ and values of V_s from Table 9. Divided and undivided circles represent experimental points for 348.16°K and 303.16°K, respectively.

near those for inert gases[6] to obtain reasonable values for the critical constants. The approximate relationship predicted between T_c and T_B is $T_c \approx 3T_B$.

In Figures 1 and 2 the molar volumes of sodium and mercury are plotted against the temperature. In Figure 3 two isotherms are shown for mercury extending up to a pressure of 12,000 kg/cm².

Thus, various thermodynamic properties can be predicted reasonably well by making use of the two parameters, n and a. The change of n at the critical point may be ascribed to a change from ions toward atoms in the solid-like degrees of freedom. The fact that the conductivities of liquid metals decrease with rising temperatures supports this viewpoint. The change from high to low n is probably gradual and continuous.

Transport Properties.—The method of significant structures applied to liquid viscosity gives the equation[23]

$$\eta = \frac{V_s}{V}\eta_s + \left(1 - \frac{V_s}{V}\right)\eta_g \tag{5}$$

where η_s and η_g are the viscosity contributions from the solid and gas-like degrees of freedom, respectively. η_g is given at moderate pressures by the kinetic theory equation[24]

$$\eta_g = \frac{m}{3\sqrt{2}\pi d^2}\left(\frac{8R}{\pi M}\right)^{1/2} T^{1/2} \tag{6}$$

where m, d, and M are the molecular mass, the effective molecular diameter, and the molecular weight, respectively. At higher densities this viscosity equation for gases must be modified.[25] Our model for doing this treats part of the degrees of freedom as solid-like. The monatomic gas-like degrees of freedom make a negligible contribution to the calculated viscosities for sodium and mercury. The diatomic molecular degrees of freedom make a small contribution, which is included.

An expression will next be obtained for η_s. The number of times per second an atom passes from one equilibrium position to another is given by[1]

$$k' = \frac{kT}{h} \cdot \frac{F^{\ddagger}}{F} e^{-\epsilon_0/kT} \tag{7}$$

where F and $F^{\ddagger}$ are the partition functions for the atom in the initial and activated states, and ϵ_0 is the activation energy. An atom in the solid-like structure can move into $n\left(\frac{V}{V_s} - 1\right)$ equilibrium positions so that the total rate per atom of jumping over barriers is

$$k'' = n\left(\frac{V}{V_s} - 1\right)k'. \tag{8}$$

For a solid-like atom

$$F = \frac{e^{E_s/RT}}{(1 - e^{-\theta/T})^3}\left[1 + n_h \cdot e^{-aE_s/n_hRT}\right] \tag{9}$$

and

$$F^{\ddagger} = \frac{e^{E_s/RT}}{(1 - e^{-\theta/T})^2}\left[1 + n_h \cdot e^{-aE_s/n_hRT}\right]. \tag{10}$$

Use of Eyring's rate process theory as applied to viscosity gives then for η_s the equation

$$\eta_s = \frac{hN}{r} \frac{1}{(1 - e^{-\theta/T})} \frac{1}{(V - V_s)} e^{bE_s/n_hRT}. \tag{11}$$

Here ϵ_0 is assumed to be of the form $\epsilon_0 = bE_s/n_hRT$ where b is a parameter to be fitted to the experimental viscosity data. r is a similar parameter which contains

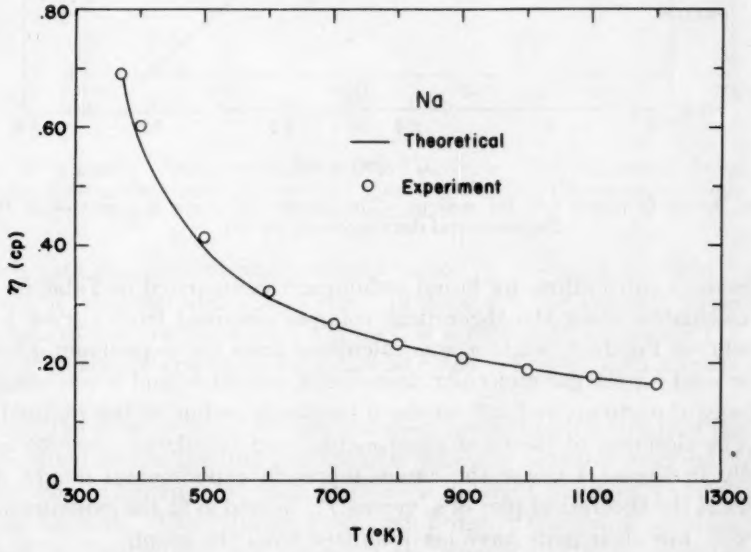

FIG. 4.—Viscosity versus temperature for sodium. The theoretical curve was calculated using volumes taken from curve 1 of Fig. 1. Experimental points are from ref. 8.

the factor n multiplied by a proper fraction to account for the fact that most of the possible jumps are in a direction skew to the direction of flow. The *a priori* estimation of r agrees quite closely with the value required in equation (11).

For high pressures a pressure term, $P\Delta V^{\ddagger}$, is added to the activation energy so that

$$\eta_s = \frac{hN}{r} \frac{1}{(1 - e^{-\theta/T})} \frac{1}{(V - V_s)} \exp\left(\frac{bE_s}{n_h RT} + \frac{P\Delta V^{\ddagger}}{RT}\right). \tag{12}$$

The self-diffusion coefficient is calculated by means of the equation[23]

$$D = \frac{kT}{\xi(V_s/N)^{1/3}\eta} \tag{13}$$

where ξ is the effective number of neighbors of a molecule lying in the same plane. For a close-packed structure this is six.

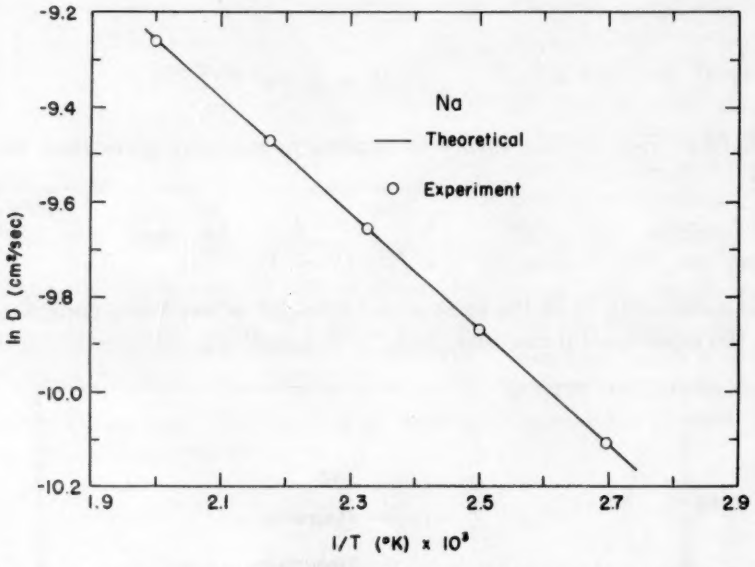

FIG. 5.—ln D versus $1/T$ for sodium. The theoretical curve is a plot of ln D_1. Experimental data are from ref. 26.

The viscosity calculations for liquid sodium are summarized in Table 5. η_1 and η_2 were calculated using the theoretical volumes obtained from curves 1 and 2, respectively, in Figure 1, while η_3 was calculated from the experimental volumes.[8] The value used for the gas molecular diameter, d, is 6.80 Å, and it was obtained by adding twice the atomic radius[22] to the interatomic radius of the diatomic molecule.[15] The closeness of the fit of experimental and calculated viscosity is shown graphically in Figure 4 where the circles represent experimental points, and the solid curve is the theoretical plot of η_1 versus T. η_2 and η_3 fit the experimental data equally well, but their plots have been omitted from the graph.

In Table 6 the values calculated for the coefficient of self-diffusion of liquid sodium are presented. D_1, D_2, and D_3 were calculated by using η_1, η_2, and η_3, re-

spectively, in equation (13). D_4 was calculated from the experimentally observed viscosities.

Figure 5 presents a plot of ln D_1 versus $1/T$. The theoretical self-diffusion coefficients agree with the observed values well within experimental error. Again for clarity D_2, D_3, and D_4 have not been plotted on the graph. Thus, for sodium the method of significant structures works very well in predicting the transport properties, viscosity and diffusion. The value of ξ is approximately six, as was to be expected.[23]

For mercury the temperature variation of the coefficients of viscosity and self-diffusion have been treated similarly. Tables 7 and 8 summarize the calculations on viscosity and self-diffusion coefficient. For mercury $d = 6.90$ Å,[15, 24] η_1 was calculated from the theoretical volumes plotted in Figure 2, and η_2 was calculated from the experimental volumes.[22] D_1 and D_2 were calculated using η_1 and η_2, and D_3 was

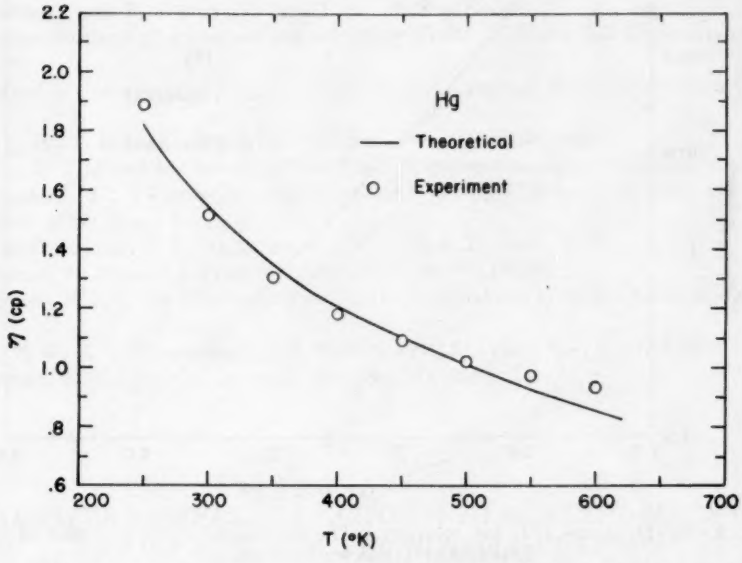

FIG. 6—Viscosity versus temperature for mercury. The theoretical curve was calculated using the theoretical volumes plotted in Fig. 2. Experimental points were taken from ref. 22.

calculated with the experimental viscosity values.[22] For η_1, $b = 0$ and $r = 2.070$; for η_2, $bE_s/R = 4.999$ and $r = 2.809$. In Figure 6 the viscosity of mercury is plotted against the temperature. Figure 7 is a plot of ln D_1 versus $1/T$. Again it is seen that the experimental data can be fitted very well with a value of about 6 for ξ.

To study the pressure effect on the transport properties the fact that the volume, V_s, of the solid at the melting point varies with the pressure must be taken into consideration. Therefore, at each pressure a different value of V_s must be used for the calculation. The value of V_s at each pressure studied is listed for mercury in Table 9.[21] Bridgman[28] has measured the viscosity of liquid mercury for the 303.16°K and 348.16°K isotherms up to a pressure of 12,000 kg/cm². η_1 calculated from the theoretical volumes plotted in Figure 3 and η_2 calculated from

experimentally observed volumes[29] are listed along with the experimental data of Bridgman[28] in Tables 10 and 11 for the two temperatures given above and for pressures up to 12,000 kg/cm². The activation volume, $\Delta V^{\ddagger}$, used is -0.62 cc, and the values used for the remaining parameters are the same as those used to calculate η_1 and η_2 in Table 7.

Nachtrieb and Petit[27] have measured the self-diffusion coefficient of liquid mercury at 303.16°K up to 8,000 kg/cm². The calculated and observed values are presented in Table 12. Again D_1 and D_2 correspond to calculations in which η_1 and η_2 of Table 10 are used, and D_3 is calculated with the experimental viscosities of Bridgman.[28] The values of ξ used are the same as those used in Table 8.

In general, the experimental data has been predicted very well for both thermodynamic and transport properties. The use of a negative activation volume for

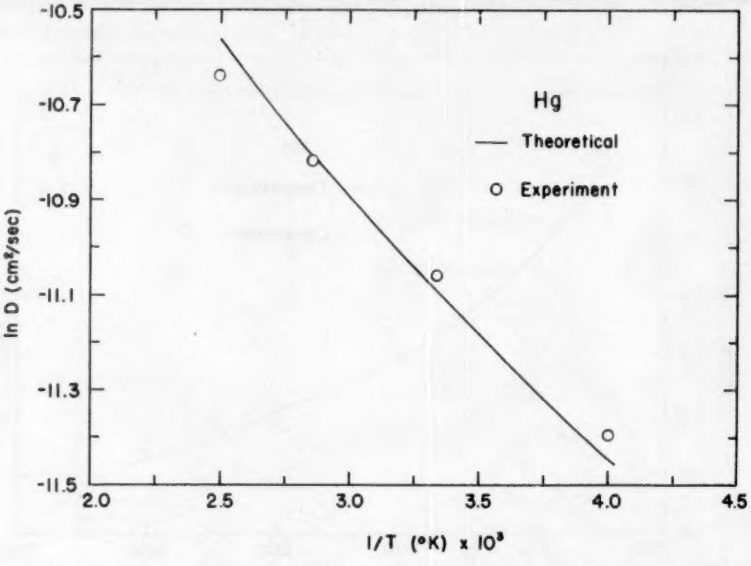

FIG. 7.—ln D versus $1/T$ for mercury. The theoretical curve is a plot of ln D_1. Experimental data are from ref. 27.

the high pressure calculations is surprising; however, this difficulty will probably disappear when the dependence of E_s on V_s is properly taken into account.

Using the significant structure theory of liquids, Blomgren[30] has very recently presented a theory of viscosity which is slightly different in approach from the one presented here.

We wish to thank the National Science Foundation and the Office of Ordnance Research, United States Army, for their generous support of this research.

[1] Glasstone, S., K. J. Laidler, and H. Eyring, *The Theory of Rate Processes* (New York: McGraw Hill Book Company, Inc., 1941), chap. 9.

[2] Kincaid, J. F., and H. Eyring, *J. Chem. Phys.*, **5**, 587 (1937).

[3] Hirschfelder, J., and H. Eyring, *J. Phys. Chem.*, **41**, 249 (1937).

[4] White, H. J., Jr., B.A. Thesis, Princeton Univ., Department of Chemistry, 1942.

[5] Walter, J., and H. Eyring, *J. Chem. Phys.*, **9**, 393 (1941).

[6] Eyring, H., T. Ree, and N. Hirai, these PROCEEDINGS, **44**, 683 (1958).

[7] Carlson, C. M., H. Eyring, and T. Ree, these PROCEEDINGS, **46**, 333 (1960).
[8] Sittig, M., *Sodium* (New York: Reinhold, 1956).
[9] Forsythe, W. E., *Smithsonian Physical Tables*, (Washington, D. C.: Smithsonian Institution, 1954). 9th rev. ed.
[10] *Gmelin Handbuch der Anorganischen Chemie, Teil 60* (Weinheim: Verlag Chemie, G. M. B. H., 1955), Kupfer A2.
[11] *Metals Handbook* (Cleveland, O.: American Society for Metals, 1939).
[12] Busey, R. H., and W. F. Giauque, *J. Am. Chem. Soc.*, **75**, 806 (1953).
[13] Hersh, H. N., *J. Am. Chem. Soc.*, **75**, 1529 (1953).
[14] Stull, D. R., and G. C. Sinke, *Thermodynamic Properties of the Elements* (Washington, D. C.: American Chemical Society, 1956).
[15] Herzberg, G., *Molecular Spectra and Molecular Structure, I. Diatomic Molecules* (New York: Van Nostrand, 1950), table 39.
[16] Ekstein, H., and M. Magat, *Compt. Rend.*, **199**, 264 (1934).
[17] Taylor, H. S., H. Eyring, and A. Sherman, *J. Chem. Phys.*, **1**, 68 (1933).
[18] Schissel, P., *J. Chem. Phys.*, **26**, 1276 (1957).
[19] Winans, J. G., and M. P. Heitz, *Z. Physik*, **135**, 406 (1953); **133**, 291 (1952).
[20] Drowart, J., and R. Honig, *J. Chem. Phys.*, **25**, 581 (1956).
[21] *American Institute of Physics Handbook* (New York: McGraw-Hill Book Company, Inc., 1957).
[22] *Handbook of Chemistry and Physics* (Cleveland, O.: Chemical Rubber Publishing Co., 1953), 35th ed.
[23] Ree, F. H., T. Ree, and H. Eyring, *Ind. Eng. Chem.*, **50**, 1036 (1958).
[24] Moore, W. J., *Physical Chemistry* (New York: Prentice-Hall, Inc., 1955), 2nd ed., chap. 7.
[25] Hamann, S. D., *Physico-Chemical Effects of Pressure* (London: Butterworth Scientific Publications, 1957), chap. 5.
[26] Meyer, R. E., and N. H. Nachtrieb, *J. Chem. Phys.*, **23**, 1851 (1955).
[27] Nachtrieb, N. H., and J. Petit, *J. Chem. Phys.*, **24**, 746 (1956).
[28] Bridgman, P. W., *The Physics of High Pressures* (London: G. Bell and Sons, Ltd., 1952), p. 346.
[29] Bett, K. E., K. E. Weale, and D. M. Newitt, *Brit. J. Appl. Phys.*, **5**, 243 (1954).
[30] Blomgren, G. E., *Ann. N. Y. Acad. Sci.*, **79**, 781 (1959).

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*COMPARATIVE COMPLEMENTATION AND GENETIC MAPS OF THE PAN-2 LOCUS IN NEUROSPORA CRASSA**

BY MARY E. CASE AND NORMAN H. GILES

DEPARTMENT OF BOTANY, JOSIAH WILLARD GIBBS RESEARCH LABORATORIES, YALE UNIVERSITY

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Present evidence in *Neurospora*[1-3] indicates that the phenomenon of complementation between allelic mutants is of widespread occurrence and that this process involves interactions occurring in the cytoplasm between defective gene products, probably polypeptides.[4] Furthermore, it is now clear that the patterns of interallelic complementation at certain loci can be described in terms of linear (one-dimensional) sequences of complementing mutants which have been designated *complementation maps*.[1, 3, 5] This discovery of a non-genetic functional test which establishes a linear sequence of allelic mutants strongly suggests that such a complementation map may reflect the linear organization of both a gene and its product. Such an hypothesis can be tested at loci where both complementation

mapping and genetic (fine-structure) mapping—based on interallelic recombination tests—can be performed, and this paper will describe the results of such a comparative study at the *pan-2* locus in *Neurospora crassa*. A brief, preliminary description of some of these results has been presented previously.[1]

Earlier studies established the occurrence of interallelic complementation as well as genetic recombination at the *pan-2* locus,[6] and subsequent investigations, which will be presented in detail here, led to the development of a complementation map of this locus. Biochemical evidence indicates that this locus in *Neurospora* controls the conversion of keto-valine to keto-pantoic acid.[6] Comparable pantothenic acid-requiring mutants in *Escherichia coli* have been shown to lack activity for a single enzyme.[7] Although similar conclusive evidence has not yet been forthcoming in tests of *Neurospora* mycelial extracts, it seems reasonable to assume that in this organism all *pan-2* mutants also lack or have impaired activity for the same single enzyme. Studies with other *Neurospora* mutants have demonstrated that interallelic complementation occurs at loci known to control the formation of single enzymes.[8-10] Hence, investigations of interallelic complementation appear to have considerable implications for an understanding of mechanisms responsible for the genetic control of protein synthesis.

TABLE 1

ORIGIN AND CLASSIFICATION OF PAN-2 MUTANTS

	Mutagen Used					
	None		X-ray		Ultraviolet	
Strains used	No. of mutants	Number complementing	No. of mutants	Number complementing	No. of mutants	Number complementing
74A	2	1	18	5	15	8
5.5A	2	2	5	2	2	0
3.1a	0	0	11	2	..	..
1167.4A	0	0	3	0	..	..
145.1A	0	0	2	1	..	..
74A-Y230-M138	2	0	13	2	0	0
Total	6	3	52	12	17	8
% complementing	..	50%	..	23%	..	47%

Complementing mutant numbers: (1) Spontaneous origin—from *74A: B3;* from *5.5A: B19, B20;* (2) X-irradiated conidia—from *74A: B5, B7, B28, B36, B39;* from *5.5A: B23, B25;* from *3.1a: B9, B10;* from *145.1A: B43;* from *74A-Y230-M138: B57, B61;* (3) Ultraviolet-treated conidia—from *74A: B65, B69, B70, B71, B72 B73, B74, B75.*

Materials and Methods.—The present studies have utilized a group of 75 pantothenic acid-requiring mutants at the *pan-2* locus (commonly referred to as "pan" B mutants) isolated by the filtration-concentration technique from untreated or from X-irradiated or ultraviolet-treated macroconidia of several different, closely related pantothenic acid-independent strains (Table 1). The standard wild type strain 74A (obtained from Dr. Patricia St. Lawrence) was inbred to produce the two additional wild type strains, 5.5A and 3.1a. The next two strains, 1167.4A and 145.1A, were obtained as pan prototrophs in asci from crosses between two *pan-2* mutants. The first occurred in an ascus in which reciprocal recombination within the *pan-2* locus had occurred, the reciprocal double pan mutant isolate being present, whereas the second isolate (145.1A) was from the ascus in which the reciprocal double pan recombinant was absent.[6] The last strain, 74A-Y230-M138, is an *ad-3B* mutant of spontaneous origin (in strain 74A), in which pan mutants were recovered as double pan-adenine mutants. Only three of the 75 mutants (B1, B34, and B72) are classi-

fiable as leaky on the basis of an ability to grow in the absence of pantothenic acid, and these grow only very slightly.

Initial tests for interallelic complementation employed mixed conidial suspensions on pantothenic acid-free agar plating medium, essentially in the manner described by de Serres.[11] Additional tests for complementation have utilized interallelic crosses of pan mutants, where the presence or absence of pseudo-wild colonies[12] serves to distinguish between complementing and noncomplementing mutant combinations. Tests for pseudo-wilds have proved especially useful in checking the results of conidial tests for the occurrence or non-occurrence of complementation between mutants closely adjacent on the complementation map; i.e., whether such mutants should be considered as affecting two distinct functional regions which are adjacent but do not overlap (complementing types) or as affecting regions which do overlap (non-complementing types). Whereas the results of complementation tests with such mutants utilizing conidia are sometimes variable (whether a given test is positive or negative appears to depend on a number of factors, such as conidial age and concentration), the distinction between the presence or absence of pseudo-wilds in critical crosses is unequivocal and regularly reproducible. Thus, in crosses between closely adjacent mutants which complement, pseudo-wilds regularly grow much more slowly than, and are easily distinguishable from, pan prototrophs arising by genetic recombination. By contrast, in crosses of mutants widely separated on the complementation map, pseudo-wilds are regularly indistinguishable phenotypically (with the exception of those mutant combinations forming temperature-sensitive heterocaryons) from homocaryotic pan prototrophs arising by genetic recombination. Pseudo-wilds can, however, be identified either by further crossing or by conidial plating tests, which serve to establish their heterocaryotic nature.

The methods used to establish the genetic relationships of complementing and non-complementing mutants in terms of a recombination map of the *pan-2* locus have been discussed in detail previously[6] and will not be elaborated here.

Results.—Complementation map of the pan-2 locus: All 75 *pan-2* mutants were tested in all pairwise combinations for their ability to complement. At least one positive response was obtained with 23 of the 75 mutants. The remaining 52 mutants failed to complement in any of the combinations tested and can be placed in a single category designated "non-complementing mutants." The failure of such mutants to exhibit interallelic complementation is not due to the presence of factors preventing heterocaryon formation, since such mutants readily form heterocaryons with other mutants blocked in different biochemical reactions. The relative frequency of complementing to non-complementing mutants appears to be related to the type of mutagen used, since mutants obtained from conidia exposed to X rays yield a smaller percentage of complementing types than do mutants from ultraviolet irradiated conidia (Table 1). Such a relationship may well result from the production by X rays (as contrasted with ultraviolet) of a larger percentage of primary mutants having extensive genetic damage, such as deletions or other associated gross chromosomal rearrangements. In the present sample of *pan-2* mutants, however, there is no clear evidence that mutants arising from X-irradiated conidia are more stable than those derived from ultraviolet-treated conidia.[6, 13]

The 23 mutants which do complement comprise 12 different groups (types)—

with from 1 to 6 mutants in each group—when classified on the basis of the pattern of complementation which they exhibit. The particular pattern of complementation of a given mutant is a genetic property of that mutant, typically exhibiting regular Mendelian segregation in crosses with wild type, or with other *pan-2* mutants.[6] Indeed, extensive crossing tests to wild type of both complementing and non-complementing *pan-2* mutants have produced no evidence that the pattern of complementation characteristic of a given mutant can be modified by recombination, provided a cross is not segregating for factors affecting heterocaryon formation. In crosses between various *pan-2* mutants, however, recombination may occur, as will be discussed later.

The relationships of the 12 different types of complementing *pan-2* mutants can be described in terms of a complementation map of the *pan-2* locus, as shown in the upper half of Figure 1. The construction of such a map depends on the

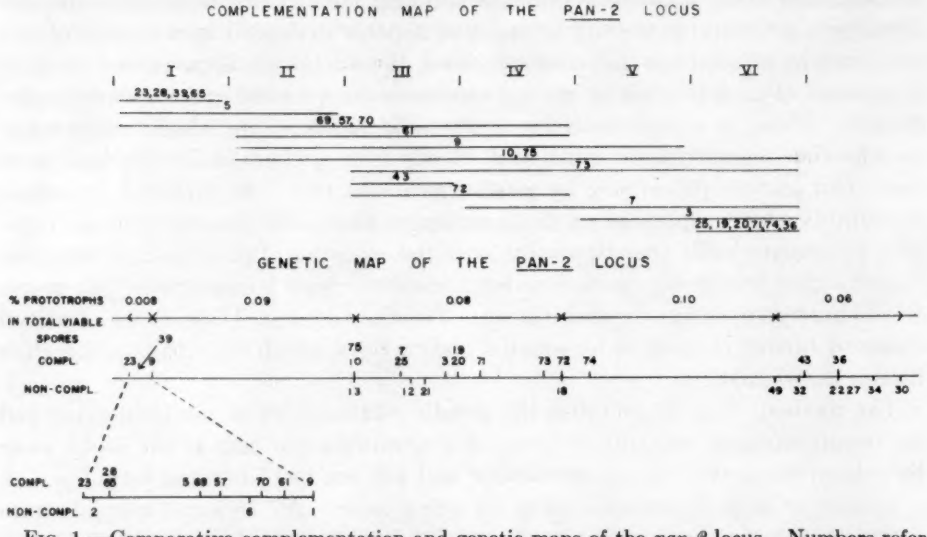

FIG. 1.—Comparative complementation and genetic maps of the *pan-2* locus. Numbers refer to individual *pan-2* mutans. On the genetic map, complementing mutants are indicated above the line, non-complementing ones below. The region between mutants *B23* and *B9* has been enlarged below the main map in order to show the positions of these mutants within this short region.

existence of complementing mutant types which fail to complement with two or more other types.[1, 3, 5] The use of such multiple "overlapping" types permits an ordering of mutants on a map, on the assumption that apparent non-functional regions in such multiple types are regularly continuous, rather than interrupted. In the present instance, the 12 types of *pan-2* mutants can be arranged in a unique linear (one-dimensional) sequence with all of the nine different multiple types represented as continuous. In the currently available group of *pan-2* mutants, 8 of the 12 types are represented by a single mutant only. Clearly, a larger sample of mutants would be highly desirable to confirm the presently indicated order. However, it is presumably significant that any change in the present order would require that certain multiple types be represented as interrupted rather than as continuous. Genetic evidence, to be presented later, supports the view that all of the multiple mutant types are single rather than double mutants.

The initial qualitative tests for complementation of *pan-2* mutants utilized mixed conidia. In general, such tests give reproducible results, and can be scored as either positive or negative. However, in certain combinations, difficulties arise, particularly in those pairs involving multiple mutants which are located in adjacent regions on the map. Such combinations are regularly slow in forming and are often erratic in repeated tests. More definitive tests for complementation between such mutants are provided by crosses where the presence or absence of psuedo-wilds can be detected, as discussed previously. Table 2 indicates the results of crosses made to test both for the expected formation of psuedo-wilds between adjacent, but non-overlapping mutants, and for the expected failure of pseudo-wild type formation between overlapping mutants. The results serve to confirm the initial heterocaryon data and to establish more firmly the boundaries of the functional units on the complementation map.

TABLE 2

EVIDENCE FOR THE PRESENCE OR ABSENCE OF COMPLEMENTATION BETWEEN VARIOUS TYPES OF COMPLEMENTING MUTANTS BASED ON TESTS FOR THE FORMATION OF PSEUDO-WILDS IN CROSSES

Complementing types	I	I-II	I-IV	II-V	II-V	II-VI	III	III-IV	IV-VI	V-VI	VI
VI (19, 20, 25, 36, 71, 74	+*	+*	+	+	+	0	+*	+*	0	0	0
V-VI (3)	+*	+	+	+	0	0	+*	+*	0	0	
IV-VI (7)	+*	+	0	0	0	0	+*	0	0		
III-IV (72)	+*	+	#	0	0	0	0	#			
III (43)	+*	+	0	0	0	0	#				
II-IV (10, 75)	+	0	0	0	0	0					
II-V (9)	+	0	0	0	0						
II-IV (61)	+	0	0	0							
I-IV (69, 70, 57)	0	0	0								
I-II (5)	0	0									
I (23, 28, 39, 65)	0										

* Pseudo-wilds occur in crosses. (In combinations marked +*, complementation responses are strong and pseudo-wilds are not phenotypically distinguishable from pan prototrophs; hence routine crossing tests were not made with all these combinations. In other combinations, complementation responses are generally weaker and pseduo-wilds can be distinguished from prototrophs. In most of these instances, crosses were made of all possible mutant combinations.)

0 Pseudo-wilds do not occur in crosses. (Tests within a particular type include both selfings and intragroup crosses.)

\# No cross obtained.

The present complementation map of the *pan-2* locus can be interpreted as indicating the existence of six functionally distinct regions at this locus. However, only three of these regions are defined by single mutant types, the existence of the other three being inferred by the occurrence of "overlaps" of multiple types. The present map thus predicts the existence of three additional single types, which presumably could be obtained in a sufficiently large sample of *pan-2* mutants. Evidence for the existence of analogous single types initially predicted by multiple type overlaps has been obtained at the *ad-4* locus.[5]

A complementation map appears to be an adequate formal representation of the qualitative relationships among complementing mutants. However, it does not directly indicate that there are also significant quantitative differences in complementation exhibited by various pairs of mutants, and that such differences can, in general, be related to the relative positions of any pair of mutants on the map. The majority of *pan-2* heterocaryons formed between mutants separated by one or more regions on the complementation map form within 24 hr after conidial

mixing and grow at 25°C on a medium lacking pantothenic acid at rates (measured as 2-day dry weights or as linear growth rates) comparable to (or in certain instances in excess of) that of wild type. However, by contrast, heterocaryons formed between mutants in adjacent regions on the map (with one exception—mutant *B43* plus mutant *B7*) form more slowly, taking 48 hr or longer before initiation of visible growth on minimal agar plates at 25°C and tend to grow more slowly.

Heterocaryons formed with mutant *B3* or *B7* are somewhat exceptional in that few achieve a wild type growth rate. However, all heterocaryons of these mutants with non-adjacent mutants form at a typically rapid rate (with the exception of the *B7-B5* pair). An additional indication of the unusual character of the *B3* and *B7* mutants is the effect of temperature on the growth rate of heterocaryons involving either of these two mutants. All heterocaryons with *B7* exhibit an unexpected type of temperature-sensitivity in that they grow more slowly at 35°C than at 25°C. Certain heterocaryons involving *B3* (those with *B23*, *B28*, *B39*, and *B65*) exhibit a similar partial temperature-sensitivity, while others (e.g., those with *B5*, *B43*, and *B72*) are completely temperature-sensitive, failing to grow at all at 35°C in the absence of pantothenic acid. The fact that mutants *B3* and *B7* share a common region (region V) on the complementation map may well be a significant factor in the similar unusual properties exhibited by their heterocaryons. Additionally, the fact that both of these mutants are stable (do not revert) and are characterized by aberrant recombination behavior (as will be discussed later) suggests that both may carry extensive genetic damage.

Genetic map of the pan-2 locus: Previous studies of *pan-2* mutants indicated the occurrence of recombination between alleles and led to the development of a linear genetic map of this locus.[6] These studies also clearly established the existence of two major groups of complementing alleles. With the development of a complementation map, it is now possible to compare the two maps and in particular to determine whether the order of mutants is the same on both maps; i.e., whether the complementation and genetic maps are "co-linear."

With this problem in mind, additional extensive studies of recombination between various *pan-2* mutants have been performed. The precise locations of all 75 mutants have not been determined as yet, but crosses involving 34 mutants have located a minimum of 27 different sites within the *pan-2* locus. Both complementing and non-complementing types have been used in crosses, a particular effort having been made to establish the positions of all the complementing mutants for comparison with the complementation map. The non-complementing mutants used for detailed mapping were selected as ones having no obvious irregularities in recombination values with adjacent markers.[6] In general, most crosses have been made between mutants which do not complement with one another; i.e., between one complementing and one non-complementing mutant, or between two non-complementing ones (including crosses of mutants within the same or overlapping functional groups). This procedure was used since crosses of complementing mutants (particularly two mutants widely spaced on the complementation map) yield numerous pseudo-wilds which in general cannot be easily distinguished phenotypically from true recombinants. All crosses were also segregating for closely-linked markers both proximal and distal to the *pan-2* locus, thus enabling mutants

to be ordered by marker behavior, as well as by additivity.[6] The resulting genetic map of the *pan-2* locus is shown in the lower half of Figure 1.

Before a comparison of the two maps is made, certain general features of the genetic map and of particular mutant crosses should be noted. Distances on the genetic map are given as "per cent prototrophs" in the total population of viable ascospores tested and are not directly equivalent to conventional recombination values, which would be obtained by doubling the present values. This correction has not been made since such a procedure assumes the regular occurrence of reciprocal recombination, which has been shown not to occur in certain interallelic *pan-2* crosses.[6]

On the genetic map diagram, complementing mutants have been placed above the line, non-complementing ones below. The region between mutants *B23* and *B9* at the left end of the map has been enlarged below the main map in order to show the positions of these mutants within this short region.

TABLE 3

RESULTS OF VARIOUS MUTANT CROSSES IN WHICH PAN PROTOTROPHS HAVE NOT BEEN OBTAINED

Cross	No. viable ascospores	No. pan prototrophs
20 ylo × 19 tryp*	544,165	0
19 ylo × 20 tryp	368,650	0
10 ylo × 75	1,075,000	0
72 × 18 tryp	391,700	0
22 ylo × 36 tryp	476,560	0
10 ylo × 13 tryp	1,613,500	0
13 ylo × 10 tryp	57,090	0
65 ylo × 28 tryp	429,640	0
65 ylo × 2 tryp	254,880	2 ylo
28 ylo × 2 tryp	105,840	0
10 ylo × 3 tryp	2,478,000	0
13 ylo × 3 tryp	114,920	0
3 ylo × 13 tryp	942,200	0
3 ylo × 75	424,000	0
7 ylo × 25 tryp	90,045	0
25 ylo × 7 tryp	1,088,713	0

* ylo = yellow, tryp = tryptophanless.

The spacing of mutants on the map is based on relative prototroph frequencies. Mutants placed at the same position are ones between which no recombination has yet been detected (Table 3). Extensive data of this kind are from the crosses of mutants *B19* and *B20*. Since these two mutants both arose spontaneously in the same experiment and behave in an identical manner in reverse mutation and complementation tests, as well as in crosses, the possibility of their common origin from a single mutational event cannot be excluded. Two additional mutants, *B10* and *B25*, which also show identical behavior in complementation tests, differ widely in origin and cannot be the result of a single mutational event. Three crosses (*B72* with *B18*, *B22* with *B36*, and *B10* with *B13*) are of particular interest, since they represent pairs in which one member is a complementing and the other a non-complementing type. In at least one of these instances, however, it is clear that the pairs of mutants differ in other respects, since *B22* is a stable mutant, while *B36* reverts. Mutants *B65* and *B28* apparently constitute another pair exhibiting no

recombination. On the basis of present data, *B28* also fails to recombine with *B2*, whereas the cross of *B65* with *B2* has yielded two recombinants. These results could be interpreted as indicating that mutant *B28* is a deletion of the type utilized in the mapping of the *r*II mutants in bacteriophage T2 by Benzer.[14] However, the number of ascospores tested in these crosses is still too small to warrant such a conclusion. In addition the behavior of mutant *B28* in crosses with *B65* and *B2* is aberrant and strongly suggests that this mutant may be associated with some type of rearrangement. In crosses of *B28* with closely adjacent mutants, no parental type prototrophs with respect to adjacent markers are recovered, whereas such types regularly occur in crosses with mutants at a distance from *B28*. Probably significant also, is the fact that *B28* is a stable mutant.

Certain crosses with two other mutants (*B3* and *B7*) have also yielded no prototrophs. However, in both these instances the available data from other crosses especially with presumptive adjacent mutants, indicate aberrant behavior of these two mutants, implying that they are genetically more complex than localized single site mutants. The proper location of *B3* is particulary difficult to establish. Despite the fact that *B3* fails to yield prototrophs in crosses with

TABLE 4

GENETIC LOCALIZATION OF PAN MUTANTS ON THE BASIS OF CROSSES TO MARKED STOCKS OF B5 AND B3

		Complementing Mutants (grouped as to origin)					Non-Complementing Mutants (grouped as to origin)				
Classification	Total	Spontaneous	X-ray	UV	Total	% Total	Spontaneous	X-ray	UV	Total	% Total
Mutants near *B5*	14	..	7	3	10	71.4%	..	4	..	4	28.6%
Mutants near *B3* or distal to *B3*	59	3	5	5	13	22.0%	3	34	9	46	78.0%
Not classified	2	..	..	..	0	....	..	2	..	2	....
Total	75	3	12	8	23	....	3	40	9	52	....

B10, *B13*, and *B75*, an examination of the frequencies and distribution of prototrophs in numerous other crosses involving *B3*, appears to place it adjacent to *B19* and *B20*. Although *B3* is placed to the left of *B19* and *B20* on the map, present data do not exclude its location to the right. As mentioned earlier, both *B3* and *B7* are stable mutants and are characterized by forming temperature-sensitive heterocaryons with other mutants.

The only complementing mutant not placed on the present genetic map is *B39*. Crosses with *B39* are characterized by low viability of ascospores, but presently available data indicate that this mutant is located at some position between *B23* and *B9*.

In addition to the crosses designated to localize particular mutants as precisely as possible, further crosses were made with all remaining *pan-2* mutants to marked stocks of both *B5* and *B3*. These crosses make it possible to assign mutants to either the left end of the map near *B5*, or to the right half (adjacent to or distal to *B3*). The results (Table 4), taken together with the earlier data, suggest a rather marked asymmetry of the genetic map of the *pan-2* locus in two respects. A disproportionately high number of mutants is concentrated in a relatively short section at the left end of the map. This group of mutants is separated by a rela-

tively long region—in which no mutants have as yet been located—from the remaining mutants, which are rather generally distributed over the right two thirds of the map (with the exception of the space between *B74* and *B49*, in which some of the mutants not yet precisely localized are apparently situated). In addition to this asymmetry in over-all mutant distribution, there is also a marked asymmetry in the distribution of complementing versus non-complementing mutants. Over 70 per cent of the mutants near the left end of the map are complementing types, whereas only about 20 per cent of those to the right on the map exhibit interallelic complementation. Nor does this asymmetry appear to be a result of a difference in mutagenic origin of the two groups of mutants, since the same general type of asymmetry characterizes mutants from ultraviolet irradiated as well as from X-irradiated conidia.

Comparison of the complementation and genetic maps: With the establishment of detailed complementation and genetic maps of the *pan-2* locus, a comparison of the two maps can now be made to determine whether they are co-linear. At first glance there appears to be, in general, a rather striking agreement between the ordering of mutants on the two maps. All mutants (with the exception of *B43*) located exclusively or primarily in regions I through III (the left half) of the complementation map, are also located near the left end of the genetic map. Furthermore, the order of mutants in this region of the genetic map agrees with the order on the complementation map, which is taken to be as follows (from left to right) for the various complementing types: I (*B23*, *B28*, *B39*, *B65*); I-II (*B5*); I-II-III-IV (*B69*, *B57*, *B70*); II-III-IV (*B61*); II-III-IV-V (*B9*). All mutants located exclusively or primarily in regions IV through VI (the right half) of the complementation map are also located in the right portion of the genetic map. There is, however, a marked asymmetry between the two maps, since all mutants in regions I through III(the left 50%) of the complementation map (again with the exception of *B43*) are confined to a region whose length is less than 2.5 per cent of the genetic map, whereas mutants in the right 50 per cent of the complementation map are located in a region which comprises approximately 70 per cent of the genetic map. No mutants, either complementing or non-complementing, have as yet been located in the space between these two regions on the genetic map. These two major groupings of complementing mutants correspond to the "*B5*" and "*B3*" groups detected in the first studies of complementation at the *pan-2* locus.[6]

Although there is a striking general correlation between the ordering of many of the mutants on the two maps, significant exceptions occur, indicating that strict co-linearity does not hold. The most significant mutants for a comparison of the two maps are those that occupy single or double regions on the complementation map, since long multiple types can only be approximately located in terms of the general distribution on the map of the several regions which they occupy. Thus the most significant mutants become those in regions I, III (*B43*), and VI, together with *B72* (III-IV). On the basis of co-linearity, mutants *B43* and *B72* should be located on the genetic map between mutants of complementation Types I and VI. However, initial recombination data from crosses of various single mutants with *B43* and *B72* located these two mutants at quite different positions among single mutants of Type VI. In order to check the positions of these two critical mutants as completely as possible, they were also crossed to three different available double

pan-2 mutants obtained by recombination within the *pan-2* locus[6] (and unpublished). The results of crosses with these double mutants, as well as with the nearest non-complementing single mutants are given in Table 5. Mutant *B72* has been somewhat difficult to work with genetically since it is leaky. In crosses, parental type ascospores of this mutant germinate rather well in the absence of pantothenic acid. However, pan prototrophs obtained in such crosses are distinguishable from the *B72* parent. The presence of pseudo-wilds in crosses of *B72* with adjacent complementing mutants was detected by using a *B72 tryp-1* parent and plating on minimal medium. By means of this procedure, only pseudo-wilds and tryptophane-independent pan prototrophs are recovered on plates.

The results of various crosses of *B43* and *B72* with other single mutants—with

TABLE 5

GENETIC LOCALIZATION OF MUTANTS B72 AND B43

Cross	No. viable ascospores	No. pan proto-trophs	Frequency of pan prototrophs	Distribution of Pan Prototrophs*				Indicated order (from marker distribution)
				Non-crossover		Crossover		
				P1	P2	++	ylo tryp	
Single × single								
72 × 18 *t*	379,800	0	0	0	0	0	0	...
12 *y* × 72 *t*	408,000	151	0.04	10	16	66	4	12–72
3 *y* × 72 *t*	58,625	6	0.01	3	3	0	0	...
22 *y* × 72 *t*	294,000	250	0.08	12	15	15	58	72–22
43 *y* × 72 *t*	579,620	475	0.08	26	10	13	51	72–43
Double × single								
5–3 *y* × 72 *t*	111,180	11	0.01	1	0	5	5	...
5–36 *y* × 72 *t*	77,180	3	0.004	0	0	2	1	...
23–36 *y* × 72 *t*	119,340	8	0.006	1	6	1	0	...
Single × single								
3 *y* × 43 *t*	105,750	301	0.28	20	0	80	0	3–43
43 *y* × 18 *t*	399,570	789	0.19	20	20	6	51	18–43
43 *y* × 49 *t*	521,220	54	0.01	7	2	5	40	49–43
32 *y* × 43 *t*	905,760	21	0.002	6	0	15	0	32–43
43 *y* × 32 *t*	379,050	11	0.003	3	2	0	6	32–43
43 *y* × 22 *t*	388,160	54	0.01	4	4	43	3	43–22
43 *y* × 27 *t*	72,420	15	0.02	4	5	5	1	43–27
43 *y* × 34 *t*	155,150	409	0.26	22	26	33	15	43–34
43 *y* × 30 *t*	203,660	320	0.15	30	6	46	18	43–30
Double × single								
5–3 *y* × 43 *t*	517,640	808	0.15	40	0	148	0	5-3-43
5–36 *y* × 43 *t*	683,120	24	0.003	8	0	16	0	...
5–36 *t* × 43 *y*	203,610	6	0.002	0	1	3	2	...
23–36 *y* × 43 *t*	679,320	16	0.003	1	3	11	1	...

y = yellow, *t* = tryptophanless.
* All prototrophs tested if less than 100 recovered; otherwise data are based on tests of a sample of 100.

respect to both prototroph frequencies and marker distribution in the two crossover categories—are generally consistent in placing these mutants as indicated on the genetic map. In addition, the results of crosses with the double mutants support the order indicated. The most significant data are those obtained in the crosses with *B43*. The *B5–B3* × *B43* cross clearly places *B43* to the right of *B3* on the basis of both prototroph frequencies and marker distribution. The *B5–B36* and *B23–B36* crosses with *B43* are also consistent on the basis of low prototroph frequencies in locating *B43* between the two members of these double mutants. Marker distribution, taken as observed, places *B43* to the right of *B36*. However, this effect is very probably related to the pecularities of recombination in such a three point intralocus cross where both conversion and negative interference

are apparently operating,[6] and the effect is probably accentuated by the close proximity of *B43* to *B36*. The quantitative results of double mutant crosses with *B72* are less conclusive, primarily because of the abnormally low recombination values characteristic of all crosses between *B3* and adjacent mutants. However, the prototroph frequency data from the cross of *B5–B3* with *B72*, compared with those from the crosses of *B5–B36* and *B23–B36* with *B72* are consistent with a localization of *B72* to the right of *B3* and to the left of *B36*.

Complementation tests utilizing double mutants: The availability of double mutants within the *pan-2* locus makes possible further significant complementation tests. The responses of various single mutant combinations permit a quantitative prediction as to the responses of various double-single mutant combinations. On the simplest hypothesis, a particular double mutant is expected to complement

TABLE 6

RESULTS OF COMPLEMENTATION TESTS UTILIZING PAN DOUBLE MUTANTS*

Combinations Tested (Singles with singles)	Type of Complementation Test		Combination Tested (Singles with doubles)	Type of Complementation Test	
	Conidial mixing	PWTS		Conidial mixing	PWTS
43 + 5	++	++	43 + 5–3	0	+
43 + 3	+++	−	43 + 5–36	0	++
43 + 23	+++	−	43 + 23–36	0	++
43 + 36	+++	−	.....	..	..
72 + 5	++	++	72 + 5–3	0	0†
72 + 3	+++	−	72 + 5–36	0	++
72 + 23	+++	−	72 + 23–36	++	++
72 + 36	+++	−	.....	..	..
61 + 5	0	0	61 + 5–3	0	0
61 + 3	++	++	61 + 5–36	0	0
61 + 23	++	++	61 + 23–36	0	0†
61 + 36	++	++	.....	..	..
9 + 5	0	0	9 + 5–3	0	0
9 + 3	++	++	9 + 5–36	0	0
9 + 23	++	++	9 + 23–26	0	0†
9 + 36	++	++	.....	..	..

* Comparisons have been made of various pairs of single mutants with various single-double mutant pairs. Evidence for complementation is based on tests for heterocaryon formation both from conidial mixing and from crosses to detect pseudo-wilds, PWTS.

Symbols used: − Complementation by heterocaryon formation following conidial mixing normal; no routine check made for PWTS. 0 No complementation detected. + Very light response; no further growth on transfer. ++ Intermediate response; further growth after transfer, but wild-type rate never attained. +++ Good response; continuing growth after transfer; rate comparable to wild type.

† Combination expected to yield PWTS on basis of absence of overlapping regions in single-double combinations.

with all single mutants with which it fails to overlap. Actually, four single mutants —*B9*, *B43*, *B72*, and *B61*—which occupy regions in the middle of the complementation map are expected to complement with one or more of the three available double mutants. Hence, tests for complementation with the double mutants were performed with all four of these single mutants, utilizing both conidial mixing tests and crosses to detect the presence of pseudo-wilds. As has already been indicated, this last test is the more sensitive and reproducible of the two tests, especially in instances where the degree of complementation is restricted.

The results of these tests, including all possible pairwise single mutant combinations as controls, are presented in Table 6. It is evident that in the double-single combinations, many more of the anticipated positive responses are obtained in the tests for pseudo-wilds than in conidial mixing tests. In fact, only

one of the conidial mixing tests involving a double gave positive results (*B23–B36* with *B72*). In several cases, unrelated biochemical "forcing" markers were employed to determine if other negative combinations would respond in conidial mixing tests. Although such heterocaryons formed on pantothenic acid-supplemented medium, the resulting heterocaryons would not grow in the absence of this supplement. These results may well be due to an inability of such heterocaryons to attain favorable nuclear ratios permitting a positive complementation response. It is perhaps not surprising that the initiation of complementing heterocaryons is most successful from pseudo-wilds where presumably two homologous chromosomes are present within the disomic nuclei of a single ascospore, a situation which leads to the formation of a heterocaryon having, at least initially, equal numbers of two types of mutant nuclei. In all instances where double-single mutant combinations gave positive responses permitting continued growth after transfer, (i.e., in all combina-

TABLE 7

RESULTS OF CIS-TRANS TESTS WITH VARIOUS PAN-2 MUTANTS*

Heterocaryons	mm/hr	Dry weight in mgs.
Controls		
74A (wild type)	3.2	35.0
B3 + *F4*	3.1	39.9
B5 + *F4*	3.1	42.0
B23 + *F4*	3.3	46.4
B36 + *F4*	3.1	41.6
Trans		
B5 + *B3*	3.0	35.2
B23 + *B3*	3.1	43.7
B5 + *B36*	3.2	45.0
B23 + *B36*	3.1	41.2
Cis		
B5-B3 + *F4* (5)†	3.1	33.5‡
B5-B36 + *F4* (2)	3.2	41.2
B5-B36 + *F4* (11)	3.2	35.9‡
B23-B36 + *F4* (7)	3.1	31.7‡

* Growth responses are given in terms of both 27-hr dry weight and linear growth rates in the absence of pantothenic acid. An *ad-4* mutant (F4) has been used as a forcing marker for the *cis* (pan double mutant combinations) hence control heterocaryons include all *pan-2-F4* pair combinations.

† Numbers in parentheses refer to particular isolates.

‡ Heterocaryon incompatibility factors are probably still influencing these growth responses.

tions except *B5–B3* with *B43*), the resulting heterocaryons were resolved and the two parental types recovered.

In three instances, double-single combinations which were expected to be positive on the basis of the absence of overlapping regions in common yielded no pseudo-wilds (*B5–B3* with *B72*; *B23–B36* with *B61*; *B23–B36* with *B9*). Moreover, microscopic examinations of plates from these crosses indicated no abortive colony formation of the type observed in crosses of *B5–B3* with *B43*. These results are perhaps not entirely unexpected in that in all these instances one or both of the pairwise combinations between a particular single mutant and the members of the double exhibit a much reduced complementation response. In addition, it is clear that even in instances where positive responses are obtained in double-single combinations, these are regularly weaker than would be anticipated from the responses of corresponding single mutant pairs. For example, both *B43* and *B72* grow at wild type rates in heterocaryons with *B23* and *B36*, yet the two corresponding double mutant combinations (*B32–B36* with *B43* and with *B72*) grow at markedly

reduced rates which are considerably less than 10 per cent of the wild type rate. Hence it is evident that when two *pan-2* mutations are simultaneously present in a given strain, they regularly interact to restrict the ability of the strain to complement to the degree expected. This interaction may result either in a weaker complementation response or in the complete absence of detectable complementation.

The availability of double mutants makes possible one further type of comparative complementation test of significance in defining the functional inter-relationships of *pan-2* mutants, i.e., the *cis-trans* test employed by Benzer[14] in defining a cistron. In this test, growth (measured as 27-hr dry weight or as linear growth rate) in the absence of pantothenic acid has been used as the measure of function. In making the *cis* comparison (double pan mutant against wild type at the *pan-2* locus) an *ad-4* mutant (*74A-Y112-M160=F4*) has been utilized as a forcing marker. Consequently an additional set of control heterocaryons has been made between this mutant and the single pan mutants tested. The results of these tests are given in Table 7. The data indicate that all the *trans* combinations tested are equal (or superior) in growth to comparable *cis* combinations, thus providing evidence for functional independence of such single mutants. The reduced dry weights characteristic of certain *cis* combinations (e.g., *B5–B36* and *B23–B36*) probably indicate the presence of genetic factors restricting heterocaryon compatibility (introduced during crossing) rather than an inherent superiority of certain *trans* combinations.

Discussion.—The mechanism of interallelic complementation: The characteristics of complementation between alleles at the *pan-2* locus are fully compatible with the hypothesis that this process involves interactions occurring in the cytoplasm of the heterocaryon between two types of differentially defective gene products derived from two types of differentially defective *pan-2* genes. Although present evidence from *Neurospora* has not yet conclusively established that the *pan-2* locus controls the synthesis of a single enzyme, such an assumption appears reasonable. Since recent *in vitro* studies with mutants at other loci known to control single enzymes make it highly probable that interallelic complementation occurs at the polypeptide level,[4, 15] the further assumption can be made that a similar situation exists at the *pan-2* locus. The actual mechanism of complementation is, however, not yet clear, but could include one or more of the following types of interactions between polypeptides: (1) Dissociation and recombination involving two or more distinct, differentially defective, polypeptide chains of a single protein, as is known to occur in the case of hemoglobin.[16] (2) The union of two different incomplete individually non-functional parts of a single polypeptide chain resulting in the restoration of enzymatic function, as has been shown to occur in the case of ribonuclease.[17] (3) Some type of cooperative interaction involving two differentially defective, polypeptide chains in an enzyme polymer molecule, as suggested by Brenner (unpublished).

The first of these mechanisms has been used in an attempt to explain complementation between mutants at the *ad-4* locus lacking activity for the enzyme adenylosuccinase,[4, 5] particularly on the basis of the maximum level of enzyme found in heterocaryons. However, the apparent presence of numerous distinct functional units at the *ad-4* locus, as at the *pan-2* locus, introduces difficulties for a simple dissociation-recombination hypothesis. Additionally, the simplest form of this hypothesis would apparently require that the enzyme present in complementing

heterocaryons be identical with that in wild type. Recent evidence indicates, however, that the adenylosuccinase present in at least some complementing heterocaryons (those which are temperature-sensitive in their growth response) is quite different from that in wild type.[18] By analogy, a similar situation may well exist in the temperature-sensitive heterocaryons involving *pan-2* mutants.

The second mechanism appears to be incompatible with present evidence from the *pan-2* locus concerning the distribution of complementing mutants on both the complementation and genetic maps. As yet no conclusion can be reached concerning the possible role of enzyme polymers in complementation, since this model has not yet been formulated in precise terms, nor has the structure been determined for any enzyme molecule known to be involved in complementation.

The genetic map of the *pan-2* locus provides significant information for interpreting the large class of "non-complementing" mutants. Formally such mutants could be represented as continuous across all six regions of the complementation map, and might be interpreted as large deletions of the entire *pan-2* locus. Such is clearly not a proper interpretation, however, since many non-complementing *pan-2* mutants are capable of reverse mutation,[6] as is true of similar mutants at other loci.[1, 3, 19] Additionally, these mutants occur at numerous different localized mutational sites throughout the *pan-2* locus, although in the present sample a larger percentage is concentrated near the right end of the map. Perhaps the simplest present interpretation of such mutants is that they make no protein related to the normal enzyme protein controlled by a particular locus, i.e., the mutational change results in the presence of nonsense DNA at the locus.[20] Thus they may be analogous to non-CRM-forming mutants at the loci in *Neurospora* and *E. coli*.[15] Alternatively, such mutants may make a protein which has been changed to such an extent that it is completely incapable of interacting, even with other differentially defective related proteins. The possibility that at least some non-complementing *pan-2* mutants make some enzyme-related protein is supported by the evidence that at least one such mutant (*B1*) is slightly leaky.

The genetic map of the *pan-2* locus also provides some evidence as to the nature of long multiply-complementing mutant types. Again, most of these cannot represent deletions, since most revert, nor are they multiple site mutants on the basis of their localization on the genetic map. An explanation for the "spreading effect" of such mutants is not obvious. One possibility may be that the mutational changes characteristic of such mutants result in mutant enzyme molecules which are incapable of interacting with long, continuous neighboring regions of homologous monomer units in an enzyme polymer.

The genetic map of the *pan-2* locus also indicates that mutants of the same complementation type need not be located at the same site. In fact such mutants may be very widely separated on the genetic map; e.g., mutants *B25* and *B36*. Thus the ability of two mutants to exhibit genetic recombination clearly does not make them capable of exhibiting cytoplasmic interactions resulting in complementation, at least to such a degree that detectable growth in the absence of pantothenic acid can occur. There is also evidence that certain pairs of mutants which are relatively closely adjacent on the genetic map may exhibit a pronounced ability to complement; e.g., *B43* and *B36* or *B72* and *B74*. To date, no pair of mutants at the same site which can complement one another has been obtained, although

three apparently non-recombining pairs exist which include one complementing and one non-complementing member. Additional genetic evidence will be required to determine whether such pairs are indeed incapable of being separated by recombination.

The problem of co-linearity of the complementation and genetic maps: In most current hypotheses of gene action,[21] the base sequence of a particular segment of DNA is assumed to serve as the code (presumably by way of an intermediate RNA template) for a corresponding sequence of amino acids in a polypeptide chain, which then undergoes folding to give the tertiary structure of the final functional protein molecule. On this view, the order and relative positions of mutations on a genetic map should correspond to the order and relative positions of corresponding altered amino acids along the polypeptide chain; i.e., the two sequences should be "co-linear."

If the process of interallelic complementation involves interactions between extended polypeptide chains, then co-linearity of the genetic and complementation maps might be anticipated if on the enzyme polymer hypothesis altered amino acids located in different parts of two otherwise identical unaltered chains (each derived from one of the two different mutant nuclei in a complementing heterocaryon) could interact to produce a functional product. An alternative possibility would be that complementation involves interactions between altered polypeptide molecules which are completely or partially folded. In such a situation, exceptions to co-linearity might be anticipated, since complementation would involve interactions between three-dimensional rather than one-dimensional structures. The exceptions to co-linearity between the two maps of the *pan-2* locus may result from a situation of this kind. In the present instance, the marked co-linearity between the left ends of the two maps is quite striking and could reflect an absence of pronounced folding in one of the terminal regions of this particular enzyme protein. However, an alternative possibility might be that complementation can occur only between mutants in this region which produce altered polypeptides characterized by an absence of normal tertiary structure in the region.

Complications in making comparisons of complementation and genetic maps could arise if certain mutants are associated with chromosomal rearrangements. Although there is no evidence for such an association in the case of mutants *B43* and *B72*, it should be recalled that *B43* is of exceptional origin in being derived from X-irradiated conidia of a pan prototroph (strain 145.1A) which itself arose by "gene conversion." It is evident that additional *pan-2* mutants (preferable of spontaneous origin) localized in single regions of the complementation map would be most useful in further tests of co-linearity between the genetic and complementation maps of this locus.

The apparent one-dimensional character of complementation maps may well be subject to exceptions, although present evidence from the *pan-2* and other loci[3, 5] suggests that such exceptions are infrequent. Clearly, a very large number of complementing allelic mutants is needed to define adequately the character of a given complementation map, particularly in those instances where the presence of several functional regions is indicated.

Functional units at the pan-2 locus: The discovery that interallelic complementation is of widespread occurrence, together with the evidence that this phenomenon

can often be described in terms of complementation maps consisting of several distinct regions, raises major problems in defining a genetic unit in terms of function.

The operational test for complementation used in *Neurospora* is the same *cis-trans* test employed by Benzer[14] in defining the "cistron" as a unit of function. Indeed, in the present studies at the *pan-2* locus, the complete test can be performed with certain mutant combinations, since mutants can be compared in the *cis* as well as in the *trans* arrangement. Such comparisons indicate complete functional independence of various pairs of mutants on the basis of the ability of such pairs in the *trans* arrangement to grow in the absence of pantothenic acid at rates equivalent to their rates of growth in the *cis* configuration. On the basis of such evidence, mutants in at least three regions (I, III, and VI) of the complementation map may be considered to represent three distinct functional groups, as defined by the *cis-trans* test. These groups should correspond to three groups of mutants in three limited continuous segments, or "cistrons," of the genetic map of the *pan-2* locus. However, this expectation is not met, since, although the mutants in region I of the complementation map occur together in one region of the genetic map, mutant *B43* (in region III) is located on the genetic map between mutants grouped in region VI of the complementation map.

Further evidence of unexpected complexities of applying the *cis-trans* test is provided by the existence of multiple (overlapping) complementing types, together with instances of partial rather than complete complementation. Perhaps most significant of all is the evidence that at the *pan-2* locus, as well as at several other loci where complementation occurs, the majority of mutants fail to exhibit any interallelic complementation. Such mutants do, however, regularly complement with other groups of mutants known to be blocked in different reactions and to be located at other loci. The existence of such non-complementing alleles located at numerous different mutational sites within a locus may well prove to be the most significant single indication derived from non-biochemical studies of the fundamental functional unity of a given group of mutants.

Tests for different functional groups of *pan-2* mutants have employed growth responses only, since a satisfactory enzymatic assay for the reaction blocked in these mutants is not yet available in *Neurospora*. Enzymatic tests can, however, be performed at the *ad-4* locus, where previous studies have developed a complementation map similar to that at the *pan-2* locus. In this instance, individual *ad-4* alleles are known to lack activity for a single enzyme—adenylosuccinase. As at the *pan-2* locus, various pairs of mutants can complement in the *trans* configuration and grow in the absence of adenine at rates equivalent to wild type. Such results provide evidence for several groups of functionally distinguishable mutants, which have been tentatively referred to as representing several "cistrons" at the *ad-4* locus.[4, 5] However, complexities similar to those among *pan-2* alleles occur in the complementation relationships at the *ad-4* locus, including the occurrence of non-complementing types. In addition, enzyme assay data have shown that complementing heterocaryons (*trans* configuration) have no more than half the adenylosuccinase activity expected of *cis* heterocaryons (on the assumption that such *cis* heterocaryons should have activities similar to those found in *ad-4*/*ad-4*$^+$ heterocaryons), despite the fact that *trans* heterocaryons grow at rates equivalent to wild type.

Thus, although the *cis-trans* test based on growth responses provides evidence of functional independence of certain groups of allelic *ad-4* mutants, biochemical evidence (based on quantitative assays and qualitative behavior of adenylosuccinase in complementing heterocaryons) indicates functional relatedness.

Clearly, present results of interallelic complementation studies in *Neurospora* demonstrate that considerable complexities exist. Hence attempts to classify functional groupings of related (isolocal) mutants in terms of "cistrons" appear premature. In fact the over-all evidence appears to indicate that the application of the *cis-trans* test in defining a "cistron" requires certain qualifications and restrictions. In his studies of *r*II mutants in bacteriophage, Benzer[14, 22] employed almost exclusively non-leaky, stable mutants, many of which behave genetically as deletions. On the basis of these mutants, evidence has been obtained by the *cis-trans* test for only two groups of functionally independent types, corresponding genetically to the A and B cistrons. However, the use of such a specifically selected group of *r*II mutants, although clearly desirable for recombination tests, may well place serious restrictions on the detection of functional interactions by means of the *cis-trans* test. Such *r*II mutants could well include only types of A and B mutants which are entirely (or largely) incapable of exhibiting intragroup functional interactions, and could thus correspond to *pan-2* alleles occurring in the non-complementing category. On this view, the *pan-2* locus, as well as the *ad-4* locus, would correspond to single "cistrons," as this term is employed in describing the A and B groups of mutants in the *r*II region. The occurrence in T4 of two adjacent genetic regions containing two functionally related, but distinguishable, groups of mutants would thus be more comparable in *Neurospora* to the situation in the *ad-3* region in linkage group I. This region is composed of two functionally quite distinct groups of mutants (*ad-3A* and *ad-3B*) corresponding to two adjacent chromosomal segments.[10] Complementation between members of the two different groups is the general rule, whereas complementation between members of the same group, although relatively rare, does occur (de Serres, unpublished). An alternative interpretation of the *r*II results would be that interactions between intracistron mutants leading to partial or complete restoration of function cannot occur regardless of the type of mutational changes involved, because of the chemical nature of the products of these segments. The existence of loci in *Neurospora* at which interallelic complementation does not occur is still a distinct possibility.[3]

If, indeed, genetic loci in *Neurospora* such as the *pan-2* and *ad-4* are to be considered unitary in some basic functional sense, such as in their control over the synthesis of a particular enzyme protein, and hence are to be considered equivalent to single cistrons, then the results of interallelic complementation studies indicate that the *cis-trans* test can still be employed in the analysis of intracistron mutant relationships. A proper interpretation of the nature and significance of the functional subunits which such studies have demonstrated must await further evidence.

Summary.- -The present study has utilized 75 mutants at the *pan-2* locus in *Neurospora crassa*. This locus controls the conversion, in the biosynthesis of pantothenic acid, of keto-valine to keto-pantoic acid, a reaction presumably catalyzed by a single enzyme in this organism. All 75 mutants were tested in all possible pair-wise combinations for their ability to complement in heterocaryons. Twenty-three of the mutants gave at least one positive response, the remaining 52 failing to

complement in any combination. The 23 complementing mutants comprise 12 different groups (type) and these 12 types can be arranged in a linear (one-dimensional) sequence to form a complementation map of the *pan-2* locus.

The relationships of mutants on this complementation map have been compared with their relationships on a genetic map of the locus derived from recombinational analyses. Although there is, in general, a rather marked agreement in the ordering of mutants on the two maps, certain marked exceptions to complete "co-linearity" occur.

Various possible interpretations of interallelic complementation and of the relationship of this phenomenon to genetic fine structure are discussed. Particular consideration is given to the characteristics of interallelic complementation as they relate to the problem of defining a genetic unit of function.

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[1] Giles, N. H., *Proc. X Intern. Cong. Genetics*, **1**, 261 (1958).

[2] Fincham, J. R. S., *Proc. X Intern. Cong. Genetics*, **1**, 355 (1958).

[3] Catcheside, D. G., and A. Overton, *Cold Spring Harbor Symposia Quant. Biol.*, **23**, 137 (1958).

[4] Woodward, D. O., these PROCEEDINGS, **45**, 846 (1959).

[5] Woodward, D. O., C. W. H. Partridge, and N. H. Giles, these PROCEEDINGS, **44**, 1237 (1958).

[6] Case, M. E., and N. H. Giles, *Cold Spring Harbor Symposia Quant. Biol.*, **23**, 119 (1958).

[7] McIntosh, E. N., M. Purko, and W. A. Wood, *J. Biol. Chem.*, **228**, 449 (1957).

[8] Giles, N. H., C. W. H. Partridge, and N. Nelson, *Proc. Internat. Genet. Sympos. Suppl. to Cytologia*, p. 543 (1956); *idem*, these PROCEEDINGS, **43**, 305 (1957).

[9] Fincham, J. R. S., and J. A. Pateman, *Nature*, **179**, 741 (1957).

[10] Lacy, A. M., and D. M. Bonner, *Proc. X Intern. Cong. Genetics*, **2**, 157 (1958).

[11] de Serres, F. J., *Genetics*, **41**, 668 (1956).

[12] Pittenger, T., *Genetics*, **39**, 326 (1954).

[13] de Serres, F. J., *Genetics*, **43**, 187 (1958).

[14] Benzer, S., "The Elementary Units of Heredity," in *The Chemical Basis of Heredity*, ed. W. D. McElroy and B. Glass (Baltimore: The Johns Hopkins Press, 1957).

[15] Yanofsky, C., and I. P. Crawford, these PROCEEDINGS, **45**, 1016 (1959).

[16] Singer, S. J., and H. A. Itano, these PROCEEDINGS, **45**, 174 (1959).

[17] Richards, F. M., these PROCEEDINGS, **44**, 162 (1958).

[18] Partridge, C. W. H., and N. H. Giles (unpublished).

[19] Woodward, D. O., and C. W. H. Partridge, and N. H. Giles, *Genetics* (in press).

[20] Levinthal, C., *Reviews of Modern Physics*, **31**, 249 (1959).

[21] Crick, R. H. C., *Symp. Soc. Exptl. Biol.*, **12**, 138 (1958).

[22] Benzer, S., these PROCEEDINGS, **45**, 1607 (1959).

*AN ENZYMATIC DIFFERENCE AMONG PYR-3 MUTANTS OF NEUROSPORA CRASSA**

By Rowland H. Davis

KERCKHOFF LABORATORIES OF BIOLOGY, CALIFORNIA INSTITUTE OF TECHNOLOGY

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The *pyrimidine-3* mutants of *Neurospora crassa* have received considerable attention in the past. Mitchell and Mitchell[1] described experiments, using *pyr-3a* (37301), *3b* (37815; temperature sensitive), and *3d* (45502), which indicated that the *pyr-3a* and *3b* mutations represent a genetic unit distinct from *3d*. The evidence for this view was that pyrimidine-independent heterocaryons could be formed by the combination of *pyr-3a* or *3b* with *3d;* that *3d*, unlike *3a* or *3b*, was unaffected by a suppressor, *s;* and that crosses between *3a* or *3b* and *3d* gave rise to a few (less than 0.01 per cent) wild type progeny. The segregation of a closely linked colonial marker (*co*), however, suggested that the wild type progeny arose through a mechanism different from simple reciprocal chromosome exchange during meiosis.

Suyama *et al.*[2] have confirmed and extended these data with a large series of independently isolated *pyr-3* mutants. Their work indicates that the *pyr-3* locus consists, on the basis of heterocaryon complementation, of two functional units, and that a large portion of the wild type progeny arising from intercrosses of mutants can be ascribed to a mechanism ("gene conversion") which does not involve the recombination of closely linked genetic markers. All of the *pyr-3* mutants, including both functional units revealed by heterocaryon complementation, appear to be arranged in a continuous, linear order, and reasonable additivity of distances prevails in the genetic map. The maximum percentage of prototrophs from crosses of mutants was approximately 0.02 per cent, which indicated a very short length of the *pyr-3* complex. (The *pyr-3d* mutant is reported to be identical with a mutant, KS36, in one of the functional units described by Suyama *et al.*;[2] *pyr-3a* and *3b* presumably represent the other unit.)

Because the patterns of heterocaryon complementation or suppressor relationship observed among *pyr-3* mutants has been observed in other mutant series concerned with one[3–6] or with more than one enzyme, it cannot be decided with the evidence available whether or not all *pyr-3* mutations affect a single metabolic function.

This investigation was designed to relate enzymatic features of the *pyr-3* mutants to the implied physiological differences between them and to the genetic structure of the *pyr-3* locus. Preliminary data have indicated that *pyr-3a*, *3b*, and *3d*, like the wild type strain, all contain the series of enzymes required for the transformation of ureido-succinic acid (US; = carbamyl aspartic acid) to uridylic acid via the orotic acid pathway, and that deficiencies in the activity of certain of these enzymes are responsible for the pyrimidine requirement of other *pyr* mutants.[7] US does not satisfy the nutritional requirement of any of the *pyr-3* mutants. The preliminary enzymatic studies, however, were considered compelling evidence that the metabolic block in *pyr-3* mutants concerned the synthesis of US or earlier steps of pyrimidine metabolism.[8] Attention was therefore directed to the synthesis of

US in *pyr-3* mutants; this study deals with the enzyme, aspartic carbamyl transferase, which catalyzes the following reaction:

L-aspartic acid + carbamyl phosphate → L-US + inorganic phosphate.

This enzyme has previously been demonstrated in *E. coli*[9, 10] and higher organisms.[11] The data presented here indicate a fundamental difference between *pyr-3d* on the one hand, and *pyr-3a*, *3b*, and wild type on the other.

Materials and Methods.—Cultures of *pyr-3a*, *3b*, *3d*, and of wild type 5297 were obtained from the culture collection of the California Institute of Technology. *Pyr-3b* requires a pyrimidine as a nutritional supplement only at temperatures higher than 32°C.[1]

Mycelia were grown for 18 to 24 hr, with shaking, in 2,500 ml culture flasks containing 700 ml minimal medium. Uridine (50 μg/ml) was used as a nutritional supplement for the pyrimidine mutants. The mycelia were harvested, washed, dried in a Buchner funnel, and dry powders were prepared after the pads were ground in cold acetone.

The extracts for enzyme work were prepared by extracting three grams of the powder twice with 20 ml 0.02 *M* tris(hydroxymethyl)-aminomethane-acetic acid (Tris-acetate) buffer, pH 8.0. The extracts were combined and acetone fractionation was applied. A precipitate which formed between 33 and 60 per cent acetone was collected by centrifugation, redissolved in 0.005 *M* buffer, pH 8.0, and dialyzed against the same buffer in small diameter tubing for 6 hr with stirring. Up to 15 per cent of the activity was lost on dialysis. A sevenfold purification could be achieved by adding aged calcium phosphate gel until the transferase activity began to decline. For most experiments, the dialyzed acetone fraction, which contained from 10 to 15 mg protein per ml, was used.

Carbamyl phosphate (CAP) was prepared by the method of Jones *et al.*[12] and was 96 per cent pure.

Aspartic carbamyl transferase activity was measured at 25° or 35° by the appearance of US after 15 min in the following reaction mixture: 10 μmoles CAP, 40 μmoles K^+ L-aspartate, 250 μmoles glycine-NaOH buffer, pH 9.1, and 0.2 to 0.4 ml of the dialyzed acetone fraction; final volume = 3.25 ml. The reaction was stopped by the addition of 0.5 ml of 2 *M* perchloric acid. After centrifuging the mixtures, 3 ml were passed quickly through a Dowex-50W column (1 × 1 cm, H^+ form), the column was rinsed with an equal volume of water,[10] and the eluate was tested by the procedure of Koritz and Cohen.[13] Inorganic phosphate was measured directly on cold perchloric acid-precipitated reaction mixtures by the method of Lowry and Lopez.[14]

Aspartic Carbamyl Transferase from Wild Type.—Wild type extracts displayed aspartic carbamyl transferase activity, and the activity was directly proportional to the amount of extract in the reaction mixtures. US was identified as a product of the reaction by chromatography[15] and by the method of Reichard and Hanshoff,[9] which involves isotope dilution and recrystallization of US to constant specific activity. D-aspartate would not serve as a substrate for the reaction. The appearance of phosphate is stoichiometrically equivalent to the US formed if the aspartate-independent breakdown of CAP is taken into account: in two experi-

ments, the ratio of μmoles phosphate to μmoles US was 1.17 to 1.09 and 0.85 to 0.84.

No cofactor requirement for the reaction could be detected. The activity was not inhibited by cyanide (0.01 *M*), arsenate (0.06 *M*), fluoride (0.01 *M*), EDTA (0.05 *M*), phosphate (0.2 *M*) or D-aspartate (0.01 *M*), but it was inhibited by iodoacetate and *p*-chloromercuribenzoate (90 per cent inhibition with 10^{-4} *M* PCMB). The inhibition brought about by PCMB was reversible to a great extent by glutathione. These results suggest that the activity of the enzyme is dependent upon sulfhydril groups; the enzyme thus resembles the equivalent transferase isolated from *E. coli*.[9] Other data pertaining to the wild type *Neurospora* enzyme are presented in the following section.

Comparison of Mutant and Wild Type Extracts.—Extracts of *pyr-3a* mycelia contained greater aspartic carbamyl transferase activity than wild type extracts.

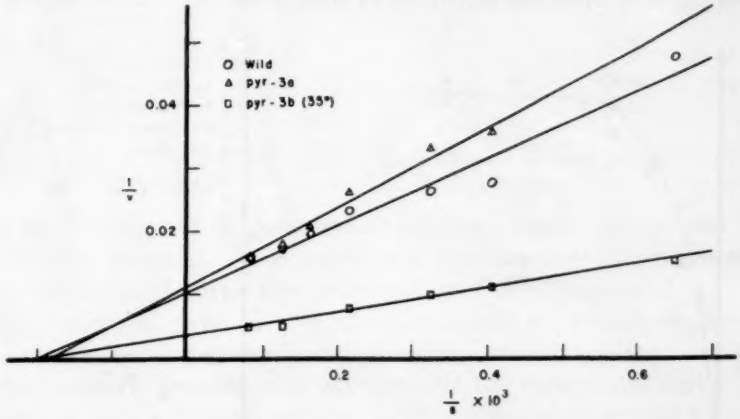

FIG. 1.—Lineweaver–Burk plot of aspartic carbamyl transferase reaction velocity, measured by the appearance of US, as a function of aspartate concentration. The standard conditions were used, except that the aspartate concentration (molar) was varied. The reactions were carried out at 25°C in the case of wild type and *pyr-3a*, and at 35°C in the case of *pyr-3b*. The lines were fitted by the method of least squares, and K_m values (see text) were derived from the X-intercepts of the least squares regressions.

Similar extracts of *pyr-3b*, grown either at 25°C or at 35°C, were active when the reaction was carried out at 25°C or at 35°C, and the relationship of the activities at the two reaction temperatures was much the same as that observed in wild type extracts (Table 1).

A further comparison of wild type and mutants was made in regard to the effects of changes in substrate concentration and the pH of the reaction mixture. By means of a Lineweaver-Burk analysis[16] (Fig. 1), the derived K_m values for aspartate for the enzymes of wild type, *pyr-3a*, and *pyr-3b* (the reaction was carried out at 35°C in the case of *pyr-3b*) were 5.0, 5.75, and 4.9×10^{-3} *M*, respectively. Moreover, no great differences in the dependence of the reaction on CAP concentration or pH could be observed in extracts of wild type and *pyr-3a* (Fig. 2).

In contrast to extracts of *pyr-3a* and *3b*, those of the mutant *pyr-3d* were consistently inactive (Table 1); an activity of one per cent of the wild type extracts would have been detected. When an extract of *pyr-3d* was added to wild type

or *pyr-3a* extracts, the activities of the latter two were not significantly altered. This rules out, to a great extent, (*a*) a free inhibitor, (*b*) an unsatisfied cofactor requirement, and (*c*) a reaction which competes with the synthesis of US, as causal factors in the inability of *pyr-3d* to yield aspartic carbamyl transferase activity.

The relationship of the activity, or lack of it, in *pyr-3a, 3b, 3d* and wild type was the same if untreated extracts of fresh homogenized mycelia were used instead of acetone powder extracts.

Discussion.—The foregoing data clearly indicate that the *pyr-3a* and *pyr-3b* mutations are not correlated with significant changes in the enzyme aspartic carbamyl transferase with regard to differences in its activity at different temperatures, pH's, or substrate concentrations. The enzyme activity of *pyr-3d* extracts, on the other hand, has been found to be less than one per cent that of similar extracts of wild type mycelia. The enzymatic difference between the *pyr-3* mutants corresponds to that observed in studies of their genetics,[1, 2] heterocaryon comple-

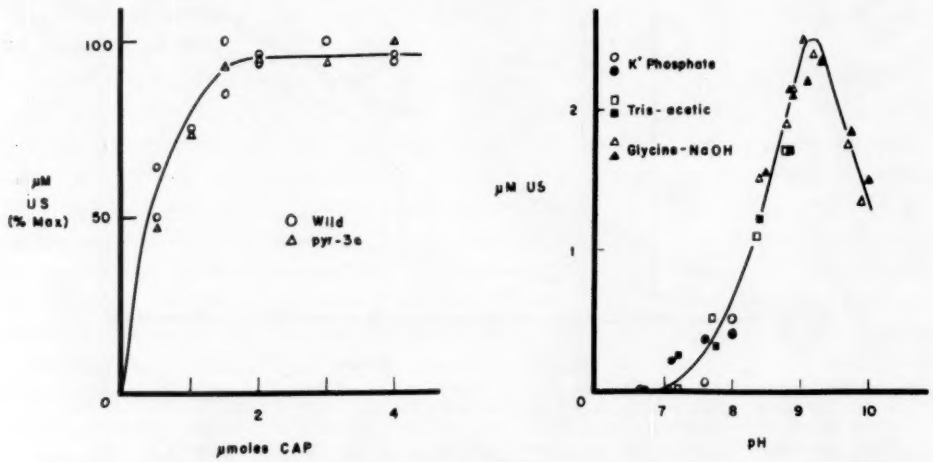

FIG. 2.—The effect of CAP concentration (μmoles per reaction mixture) (left) and *pH* (right) on aspartic carbamyl transferase activity of wild type and *pyr-3a* extracts. In the right-hand figure, solid symbols represent wild type, open symbols *pyr-3a*.

mentation,[1, 2] and suppressor response.[1] Four other pyrimidine-requiring strains presumed to represent the *pyr-3* locus, 49001, 63902, 68902, and 47102, have also been found to lack aspartic carbamyl transferase activity. (Mutant 49001 has been tested and found to resemble *pyr-3d* in its lack of response to the suppressor.[1]) It may be concluded that the lack of aspartic carbamyl transferase activity in these strains and in *pyr-3d* is directly related to the pyrimidine requirement of the mutants themselves. In light of the findings reported here, it is improbable that the series of *pyr-3* mutations directly affects only one enzyme. What has been designated as the "*pyr-3*" locus, a genetic complex covering less than (0.05) map units, is undoubtedly concerned with more than a single function.

These data further show that the inability of *pyr-3d* to grow on medium supplemented with US does not reflect the site of the metabolic block in this mutant.[17] There is, therefore, no reason to think that a similar lack of response to US by *pyr-3a* and *3b* is significant either. This suggestion, together with the preliminary

enzymatic data on *pyr-3* mutants referred to above, allow the assumption that *pyr-3a* and *3b* are blocked before the synthesis of US.

The nature of the biochemical lesion in *pyr-3a* and *3b*, however, has not yet been elucidated. In a series of experiments, Mitchell and Mitchell[18] made observations of the nutritional requirements of strains carrying any one of several arginine mutations and *pyr-3a* or *s*. Their data imply that there is a significant connection between the metabolic block in *pyr-3a* and the synthesis of arginine, and that the synthesis of arginine and pyrimidines is to some extent competitive. Such a competition may operate in the utilization of carbamyl phosphate or aspartic acid, both of which are normally required in pyrimidine and arginine synthesis. A

TABLE 1

ASPARTIC CARBAMYL TRANSFERASE ACTIVITIES OF WILD TYPE AND MUTANT EXTRACTS*

Source of Extract	μM US formed at 25°	μM US formed at 35°	Ratio: Act. 35° / Act. 25°
wild type (5297) grown at 25°	1.43	2.58	1.8
pyr-3a (37301) grown at 25°	6.20	..	..
pyr-3b (37815) grown at 25°	2.33	3.99	1.7
pyr-3b (37815) grown at 35°	1.11	2.11	1.9
pyr-3d (45502) grown at 25°	0.00	..	..

* Approximately 3 mg protein of the dialyzed acetone fraction was used in assays of all strains except *pyr-3d*, where 20 mg was used.

study of the relationship of arginine and pyrimidine synthesis in wild type and *pyr-3a* has been initiated. It is hoped that these further investigations can be profitably related to the extensive genetic analysis of Suyama *et al.*[2]

Summary.—Several pyrimidine-requiring mutants of *Neurospora crassa*, presumed to represent a single locus (*pyr-3*), have been studied in regard to the activity of aspartic carbamyl transferase of mycelia. It has been found that *pyr-3d* and certain other mutants yield no activity, while *pyr-3a* and *3b* appear to contain an enzyme indistinguishable from that demonstrated in the wild type. The results suggest that the *pyr-3* "locus" is concerned with more than a single function.

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* This work was done while the author was a regular postdoctoral fellow of the National Science Foundation at California Institute of Technology, 1958–1960.

[1] Mitchell, M. B., and H. K. Mitchell, *Genetics*, **41**, 319 (1956).

[2] Suyama, Y., K. D. Munkres, and V. W. Woodward, *Genetica*, **30**, 293 (1959).

[3] Giles, N. H., C. W. H. Partridge, and N. J. Nelson, these PROCEEDINGS, **43**, 305 (1957).

[4] Woodward, D. O., C. H. Partridge, and N. H. Giles, these PROCEEDINGS, **44**, 1237 (1958).

[5] Fincham, J. R. S., and J. A. Pateman, *Nature*, **179**, 741 (1957).

[6] Yanofsky, C., and D. M. Bonner, *Genetics*, **40**, 761 (1955).

[7] Davis, R. H. (unpublished); Tuttle, A. L., (unpublished).

[8] A similar conclusion has been reached on other grounds by Munkres, K. D., Y. Suyama, and V. W. Woodward, *J. Biol. Chem.* (in press) (cited by ref. 2), and Munkres, K. D. (personal communication).

[9] Reichard, P., and G. Hanshoff, *Acta Chem. Scand.*, **10**, 548 (1956).

[10] Yates, R. A., and A. B. Pardee, *J. Biol. Chem.*, **221**, 743 (1956).

[11] Lowenstein, J. M., and P. P. Cohen, *J. Biol. Chem.*, **220**, 57 (1956).

[12] Jones, M. E., L. Spector, and F. Lipmann, *J. Am. Chem. Soc.*, **77**, 819 (1955).

[13] Koritz, S. B., and P. P. Cohen, *J. Biol. Chem.*, **209**, 145 (1954).

[14] Lowry, O. H., and J. A. Lopez, *J. Biol. Chem.*, **162**, 421 (1946).
[15] Fink, R. M., *et al.*, *Anal. Chem.*, **28**, 4 (1956).
[16] Lineweaver, H., and D. Burk, *J. Am. Chem. Soc.*, **56**, 658 (1934).
[17] A similar finding is reported for uracil-requiring mutants of *Schizosaccharomyces pombe* by Megnet, R., *Arch. für Vererbungsforschung, Sozialanthropologie, u. Rassenhygiene*, **33**, 299 (1959).
[18] Mitchell, M. B., and H. K. Mitchell, these PROCEEDINGS, **38**, 205 (1952).

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*ANTAGONISM BETWEEN SUBSTRATE AND REPRESSOR IN CONTROLLING THE FORMATION OF A BIOSYNTHETIC ENZYME**

LUIGI GORINI

DEPARTMENT OF BACTERIOLOGY AND IMMUNOLOGY, HARVARD MEDICAL SCHOOL

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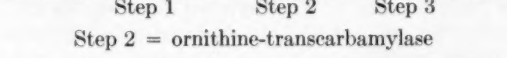
Since the formation of many degradative enzymes is induced by adding to the culture medium the corresponding substrate, it has often been suggested that the "constitutive" nature of biosynthetic enzymes depends on the endogenous formation of their substrates. It would appear possible to test this hypothesis by using a genetic block to prevent this endogenous formation. However, previous attempts to study the problem in this way[1, 2] have led to inconclusive results, since growth on the substrate was compared with growth on the end product of the biosynthetic sequence, and this end product has since been found to repress the formation of the enzyme. Furthermore, the added substrate was also serving as a source of the repressor. An additional possible complication of such experiments is formation of the substrate from the added end product, in those cases where the reactions between the two compounds are reversible.

In undertaking a reinvestigation of this problem with ornithine transcarbamylase of *Escherichia coli*, an enzyme of arginine biosynthesis which catalyzes the conversion of ornithine to citrulline, we have controlled these interfering factors by two devices: the use of a chemostat and the use of double mutants blocked both before and after the reaction under study. The chemostat made it possible to reduce repression by supplying mutants with the required arginine at low, controlled concentrations.[3] In the double mutants (Fig. 1), the earlier block



Step 2 = ornithine-transcarbamylase

FIG. 1.—Schematic pathway of arginine biosynthesis.

prevents endogenous formation of the substrate (ornithine), while the later block prevents both formation of the end product of the seqence from added substrate and formation of the substrate from added end product. In *E. coli* there appears to be no additional pathway to ornithine.[4] The study has been extended to include not only arginine-repressible strains but also strains in which arginine does not have this effect.

Bacterial Strains.—In the present experiments the wild type and mutants of different strains of *E. coli* have been studied: the W strain, in which added arginine

represses the formation of ornithine transcarbamylase, and two variants of the B strain, in which it does not. These two variant strains are: normal B, which produces low concentrations of the enzyme, and B(4S-7), derived from B^5, in which the level of the enzyme is 40 times higher.†

Table 1 contrasts the effect of added arginine on the enzyme levels in repressible strains (W and K12), which may be designated as R+ (R for repressibility), and

TABLE 1

LEVEL OF ORNITHINE-TRANSCARBAMYLASE (U/MG BACT. DRY WEIGHT) AT DIFFERENT LEVELS OF ARGININE POOL IN REPRESSIBLE AND NONREPRESSIBLE STRAINS

Strain	Repressibility	Enzyme Level: Arginine pool very low*	Arginine pool normal†	Arginine pool high‡
K12	+	104.0	8.0	0.5
W	+	67.0	2.0	0.1
B	−	4.0	5.0	7.0
B(4S-7)	−	140.0	132.0	160.0

* Arginine-requiring mutant, blocked in position 1 or 3, growing in a chemostat under arginine limitation.[9]
† Wild type exponentially growing in minimal medium A.
‡ Wild type or arginine auxotroph growing in excess of arginine.

in nonrepressible (R−) strains [B and B(4S-7)]. The designation of repressibility as a genetic character distinct from enzyme formation is based on the result of experiments in which mutants lacking the enzyme (E−) were derived from the nonrepressible B or B(4S-7) strains and crossed with a repressible E+ donor of the K12 strain.[5] Two classes of E+ recombinants were obtained: R+ and R−.

To obtain the desired double mutants (designated as type 1–3) of the various prototrophic strains, one-step mutants blocked somewhere before ornithine (block 1) were irradiated with ultraviolet light and second step mutants were selected which could grow on arginine but no longer on citrulline (block 3). The selection was carried out by a modification[6] of the penicillin technique which improves the recovery of rare auxotrophic mutants. The retention of block 1 in each 1–3 mutant was verified by checking the requirement of back mutants, selected on ornithine, that had lost block 3.

Growth Conditions.—The organisms were grown at 37° in mineral-glucose-citrate medium A,[7] supplemented with arginine, as necessary. The chemostat experiments followed the usual procedure.[3]

Determination of Ornithine-Transcarbamylase Activity.—Enzyme activity was determined in toluenized cells as previously reported.[8] One unit of enzyme is the amount which synthesizes 1 μmole of citrulline per hour. The figures reported in the tables are units of enzyme per mg bacteria (dry weight).

Control of Intracellular Arginine Levels.—It has been shown previously[9] that in the W strain of *E. coli* growth in the presence of exogenous arginine results in essentially complete repression of ornithine transcarbamylase, i.e., the level of this enzyme is reduced to a barely detectable value. On the other hand, the maintenance of an abnormally low intracellular arginine level, by slow growth of an arginine auxotroph in an arginine-limited chemostat, results in a concentration of the enzyme 25 to 50 times that found under "normal" conditions (i.e., in the wild type growing on minimal medium).[9] This state has been called derepression. For purposes of the present work it was necessary to see whether the chemostat

could be used to maintain arginine levels that would result in enzyme concentrations intermediate between complete repression and derepression.

In the chemostat,[3] with an arginine auxotroph and a medium in which arginine limits the density of growth, differences in the rate of flow of new medium are associated with parallel differences in the steady-state rate of growth. This remains true over a range of growth rates corresponding to division times between one hour and several hours. At higher flow rates, exceeding the division time of the organism without limitation of arginine, the population density falls progressively and arginine accumulates rapidly in the culture fluid. Under steady-state conditions, since arginine is limiting, the theory of the chemostat[3] predicts that each growth rate is associated with a characteristic extracellular (and therefore a characteristic intracellular) level of free arginine: the shorter the division time, the higher the arginine level.

It should thus be possible to run the entire gamut of intracellular arginine concentrations by varying the flow rate in the chemostat. Figure 2 shows the effects of such variation on the concentration of the enzyme in mutant 160–37 of the W strain of *E. coli*, which is blocked before ornithine. It is seen that over a range of low flow rates there is complete derepression—i.e., maximal enzyme formation. At the other extreme, at flow rates too great for arginine limitation, there is essentially complete repression, with very low levels of the enzyme similar to those seen in the wild type growing in the presence of excess arginine. At flow rates approaching the limit of the steady-state (1–1.5 hr division time) intermediate levels of the enzyme are seen. It is thus possible by use of the chemostat to maintain in an arginine auxotroph levels of the enzyme (and hence intracellular levels of arginine) similar to those produced by the wild type growing on minimal medium.

Effect of Ornithine in a Repressible Strain.—The effect of ornithine on transcarbamylase formation was studied in a type 1–3 mutant of an R+ strain. It is known[9] that in R+ strains arginine represses transcarbamylase synthesis irrespective of any genetic block interposed in the sequence of enzymes leading to arginine synthesis. In order to control the level of arginine the experiments were performed in chemostats with arginine-limiting growth. Two situations have been analyzed: partial repression and derepression. The effect of different concentrations of ornithine has been tested under each of these conditions.

To achieve the partially repressed condition, the experiment was started at a flow rate as fast as was compatible with the steady state. As is seen in Figure 2, these conditions resulted in an intracellular arginine level that repressed the enzyme content per cell to about $^1/_{20}$ of its maximum, i.e., similar to that in wild-type strain W growing exponentially in minimal medium. At the end of the experiment, in order to achieve the condition of derepression, the intracellular arginine level was lowered by halving the flow rate. As is seen in Figure 2, under these conditions a maximal level of enzyme is reached, i.e., maximal derepression. The enzyme level was determined in samples taken after each steady state was reached, i.e., 6 to 8 hours after the start of the experiment, after the addition of ornithine, and after the change in flow rate. That a steady state was reached is shown by the fact that samples taken 3 hours later gave essentially the same values.

Tables 2 and 3 give the results of two typical experiments. It is seen that under a condition of partial repression (fast flow rate) ornithine stimulates formation

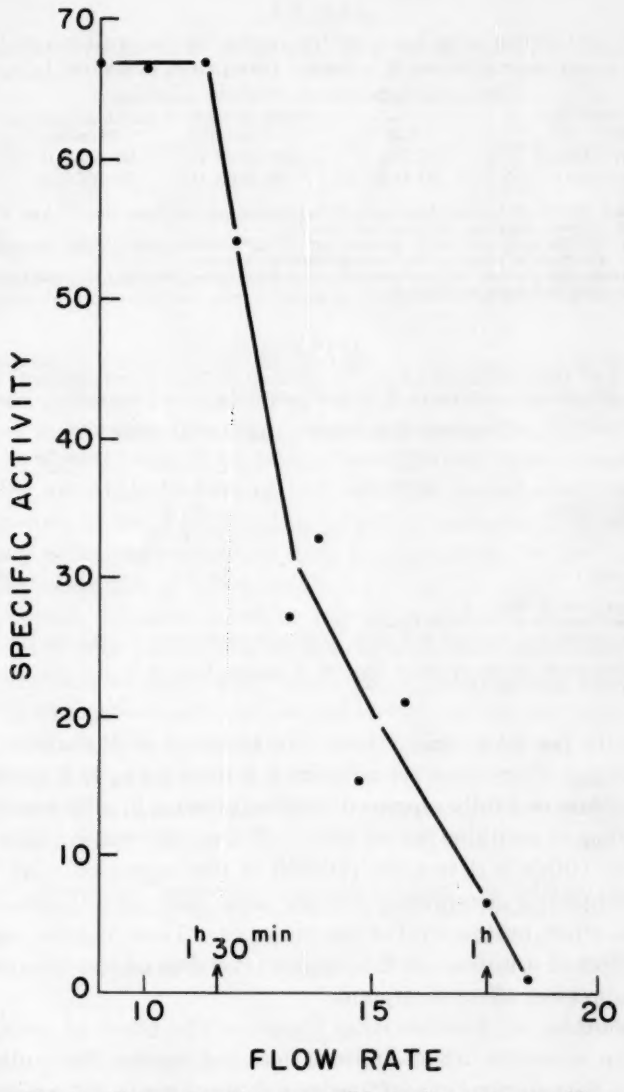


FIG. 2.—Enzyme level in a repressible strain versus rate of flow. Mutant W160-37, type 1-3, was grown in a chemostat under arginine limitation. The culture was fed with medium containing 10 mg of arginine/L at the flow rate indicated in the abscissa (ml/hr). The ordinate is specific activity: units of ornithine-transcarbamylase/mg bacteria dry weight. The arrows on the abscissa indicate the division time calculated from the flow rate (W) and the total volume of the culture (V = 25 ml) according to the formula: div. time = $(V/W) \times ln2$. Steady-state conditions are possible only with flow rates lower than 17.5 ml/hr corresponding to a division time of 1 hr.

of the enzyme.‡ Ornithine totally overcomes this degree of repression at a concentration of 15 to 20 μg/ml in the input medium. Furthermore, by comparing the two tables one realizes that the stronger the repression, the higher is the concentration of ornithine required in the input medium to yield a given level of the enzyme

TABLE 2

EFFECT OF ORNITHINE ON LEVEL OF ORNITHINE-TRANSCARBAMYLASE (U/MG BACT. DRY WEIGHT) IN AN R+ STRAIN BLOCKED IN POSITIONS 1 AND 3

Chemostat Experiment: Arginine Limiting

Flow rate	Enzyme Level at Ornithine Conc. of: 0 μg/ml	1 μg/ml	10 μg/ml	100 μg/ml
Fast (=1 hr div. time)	5.1(6.3)	21.9(22.0)	50.5(42.0)	61.5(67.0)
Slow (2 hr div. time)	50.0(60.0)	56.2(63.0)	50.5(54.0)	58.0(51.0)

Organism.—Mutant W 160-37 blocked between acetylornithine and ornithine (block 1) on which has been imposed a second block between citrulline and arginine (block 3).

Growth conditions.—Medium A with 0.1% glucose and 10 μg/ml of arginine. Four parallel chemostats with identical flow rates. Ornithine is added to the input medium at time zero.

The figures in parentheses are the levels of enzymes in samples taken 3 hr after the initial sample. They demonstrate that steady state had been established.

TABLE 3

EFFECT OF ORNITHINE ON LEVEL OF ORNITHINE-TRANSCARBAMYLASE (U/MG BACT. DRY WEIGHT) IN AN R+ STRAIN BLOCKED IN POSITIONS 1 AND 3

Chemostat Experiment: Arginine Limiting

Flow rate	Addition of Ornithine	Enzyme Level Chemostat 1	Chemostat 2
Fast (= 1 hr/div. time)		2.4(4.5)	3.5(2.5)
	→	+2μg/ml	+20μg/ml
Same rate		20.0(15.8)	42.5(47.0)
Slow (2 hr div. time)		56.0	58.0

Organism.—The same as in Table 2.

Growth Conditions.—Same medium as in Table 2.

Two parallel chemostats with identical flow rates. Ornithine is added to the input medium after equilibration of the chemostat.

The figures in parentheses are the levels of enzymes in samples taken 3 hr after the initials ample. They demonstrate that steady state had been established.

(1 μg of ornithine per ml to raise the enzyme from 5.1 to 21.9 units in Table 2, as compared with 2μg of ornithine per ml to raise it from 2.4 to 15.8 units in Table 3). In the extreme case of a fully repressed culture (growing in a flask with an excess of arginine) 1,000 μg of ornithine per ml (about 15 times the concentration of arginine) are required to obtain a detectable reversal of the repression. At the other extreme, in a completely derepressed culture (slow flow rate, Tables 2 and 3), ornithine had no effect on the level of the enzymes. These results suggest that the stimulatory effect of ornithine on the enzyme level depends on competitive antagonism to the repressive effect of arginine.

Effect of Ornithine in Nonrepressible Strains.—The effect of ornithine has also been studied in strains in which arginine does not repress the synthesis of transcarbamylase. Since control of arginine level during growth did not seem important with these strains, the experiments were carried out in flask cultures and the level of the enzyme was measured after overnight growth. However, in order to be sure that the arginine level did not affect the results, two extreme conditions have been analyzed: arginine limitation and arginine excess.

It is seen (Table 4) that with these R− strains addition of ornithine was not required for, and furthermore did not stimulate, formation of the enzyme, even in the presence of excess arginine. It should also be noted that the constant values of this enzyme as seen in these mutants under these various conditions are comparable to those observed in the prototrophic strains (Table 1) from which those double mutants were derived.

The results obtained with R+ and R− strains suggest that ornithine stimulates the formation of transcarbamylase only in the presence of arginine.

TABLE 4

EFFECT OF ORNITHINE ON LEVEL OF ORNITHINE-TRANSCARBAMYLASE (U/MG BACT. DRY WEIGHT) IN R− STRAINS BLOCKED IN POSITIONS 1 AND 3

	Enzyme Level			
	Arginine limiting*		Arginine in excess†	
In presence of ornithine:	Strain B-(1–3)	Strain B(48-7)-(1–3)	Strain B-(1–3)	Strain B(48-7)-(1–3)
0	5.2	135.0	7.5	169.9
10 μg/ml	4.9	138.0	..	...
60 μg/ml	..	...	8.0	164.0
100 μg/ml	..	...	7.2	165.0

Organisms.—Type 1–3 mutants of B and B(48-7).
Growth Conditions.—Overnight cultures, growth limited by: * Arginine (arginine 20 μg/ml); † glucose (arginine 60 μg/ml).

Nonrequirement of Ornithine in the Absence of Repression.—A double block in positions 1 and 3 should prevent endogenous formation of ornithine. However, such a mutant is ornithine-free only if block 1 is complete. Indeed, the effect of any leakage in block 1, even if undetectable by growth tests, would be enhanced by the impositions of block 3 because any ornithine formed would tend to pile up. It would therefore be desirable to obtain further evidence to determine whether or not traces of endogenous ornithine may be responsible for the formation of the enzymes under conditions of derepression.

For this purpose, mutants blocked only in position 1 and mutants blocked only in position 3 were grown in a chemostat at a slow rate under arginine limitation, and their enzyme levels were compared. Under these conditions the intracellular level of endogenous ornithine should be high in mutants blocked in position 3 and very low, if not zero, in mutants blocked in position 1.

Table 5 gives the enzyme level in Type 1 and Type 3 mutants of several strains (including R+ and R−). It is seen that in every strain under these derepressed

TABLE 5

LEVEL OF ORNITHINE-TRANSCARBAMYLASE (U/MG BACT. DRY WEIGHT) IN MUTANTS OF VARIOUS STRAINS BLOCKED IN DIFFERENT POSITIONS

		Enzyme Level		
Parent strain	Repressibility	Block 1*	Block 3*	Wild type†
W	+	65.0	62.0	...
K12	+	94.0	100.0	...
B(48-7)	−	150.0	146.0	145.0
B	−	6.0	7.0	5.0

* Cultures growing in a chemostat under arginine limitation (10 μg/ml). Division time = 4 hr.
† Overnight culture in minimal medium A.

conditions the enzyme level in mutant 1 is not significantly different from that in mutant 3. These results, together with those reported above for type 1–3 mutants, support the conclusion that in the absence of repression, whether because the strain is not repressible or because insufficient repressor is present, ornithine is deprived of any influence on the formation of transcarbamylase. Furthermore, the results presented in Table 5 show that whatever may be the factor responsible for the differences in production of this enzyme by the different strains, it is not the level of endogenous ornithine.

Discussion.—By using double mutants blocked both before and after ornithine transcarbamylase, and by controlling the arginine concentration by means of growth in a chemostat, it has been found that ornithine can reverse the repressive

effect of arginine on the formation of this enzyme. The induction by ornithine occurs only under conditions of partial repression. When repression is eliminated by sufficiently lowering the arginine concentration, or by using a nonrepressible strain, the inducing effect of ornithine is also eliminated and the concentration of the enzyme for the given strain appears to be maximal without any endogenous or added ornithine. Conversely, when repression is complete, in the presence of excess arginine, even comparatively large amounts of ornithine are without effect. The reversing effect of ornithine appears to be competitive with arginine. However, a strict test for competition was not possible because of technical difficulties in controlling intracellular arginine levels over a sufficient range.

Because the repressor and its antagonist are both provided exogenously in these doubly blocked mutants, one must consider the possibility that the observed interactions might be based simply on competition for entry into the cell. Owing to this consideration, the results of preliminary experiments performed in flask cultures were inconclusive. In these experiments the effect of ornithine on the level of transcarbamylase was studied in overnight cultures whose growth was limited by arginine. Five to six times more enzyme was found in the presence of ornithine than in its absence. However the possibility that this effect was due to arginine-ornithine competition for entry could not be ruled out.

As is shown by the following reasoning, the present experiments in the chemostat have eliminated the possibility that the inducing effect of ornithine could be due to interference with the entry of arginine. Since the growth rate of an arginine-limited culture is determined by the intracellular concentration of arginine, each steady-state flow rate determines a corresponding fixed intracellular concentration. Under ordinary conditions, in the absence of any inhibitor of entry, each steady-state intracellular concentration will correspond to a fixed extracellular concentration. When a competitive inhibitor of entry is added to the medium in a steady-state system the entry of arginine will be slowed and the cell density will decrease, until the external arginine concentration reaches a new higher level which precisely compensates for the impaired membrane transport. At this point a new steady state will be reached, with restoration of the original division time and hence the original intracellular arginine concentration. It follows that if ornithine should interfere with arginine entry it would not affect the intracellular level of arginine in these experiments, provided enough time is allowed for reaching the steady state.

Actually, at a fast flow rate the extracellular arginine is an appreciable fraction of the total amount available, and so competition for entry might be expected to cause a measurable drop in cell density. In our experiments the drop observed after addition of ornithine was never more than $^1/_{10}$ of the original density, indicating that the competition for entry is small.[§] Hence, the time allowed in these experiments (several generations) was ample for reaching the new steady state, as is verified by the essentially constant values of the enzyme found during an additional interval of 3 hrs (Tables 2 and 3).

It seems safe to conclude that the inducing of ornithine is based on competition with arginine at some site within the cell: either the site of repressor action, if arginine is the direct repressor, or the site of repressor formation, if arginine is a precursor of the direct repressor. Since transcarbamylase is necessarily present

in these experiments, the possibility cannot be rigorously excluded that citrulline rather than ornithine is the true reverser of repression. However, since the reaction is reversible, either compound is a substrate of the enzyme, and the phenomenon would still be one of induction of a biosynthetic enzyme by its substrate.

Recent work on the β-galactosidase system[10] has strongly indicated that the mode of action of the inducer is to counteract repression by an unknown compound, an inducible strain thus being actually R+ and a constitutive strain (which does not require any inducer) being R−. The present findings provide another kind of evidence, in which the repressor is known, in support of the same conclusion for another enzyme. Furthermore, by showing that a biosynthetic enzyme can respond to an inducer as well as to a repressor, these findings furnish further evidence against the earlier idea that there might be any fundamental difference in the mechanism or in the control of formation of so-called constitutive and adaptive enzymes. At the same time, since ornithine is an inducer only under conditions where arginine is acting as a repressor, these findings eliminate, at least for this system, the often-quoted speculative proposition that the constitutive nature of biosynthetic enzymes might be due to the inductive effect of the endogenously formed substrate.

Summary.—The effect of ornithine on the synthesis of ornithine-transcarbamylase has been studied in strains (R+) in which the synthesis of this enzyme is repressed by arginine and in strains (R−) in which it is not repressed.

To permit independent control of ornithine and arginine concentration, double mutants blocked both before and after this enzyme were used. Furthermore, the chemostat was used to maintain arginine levels that resulted in enzyme concentrations intermediate between complete repression and derepression.

Ornithine reversed the repressive effect of arginine in an intermediate concentration range; however, it was without effect under conditions of complete repression or derepression.

The nature of the chemostat experiment is self-correcting for arginine-ornithine competition for entrance into the cell. It therefore appears that ornithine competes directly with arginine at some site within the cell.

An analogy exists between the repressor-inducer relationship in the cases of ornithine transcarbamylase, a biosynthetic (so-called "constitutive") enzyme, and β-galactosidase, a degradative (so-called "inducible") enzyme.

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† Genetic recombination experiments, which will be discussed elsewhere, indicate that this difference in level of enzyme activity is due to the existence of a controlling factor genetically distinct from repressibility, rather than to an alternation in the efficiency of the enzyme formed. The action of ornithine has been tested in both variant strains in order to test a possible interaction of ornithine with such a control mechanism.

‡ Since it is difficult to maintain rigorous steady-state conditions in the chemostat at the fast flow rates of these experiments, which approach the threshold for escape from the steady state, one must consider the possibility that the apparent effect of ornithine on the level of the enzyme might be due, in reality, to uncontrolled fluctuations in the flow rate. However, one can estimate from Figure 2 that the flow rate would have to change by a factor of 1.5 in order to obtain the

maximal effect ascribed to ornithine in Tables 2 and 3. This change would far exceed the possible error in the control of flow rate in these experiments. It follows that the observed inducing effect of ornithine is a real one, whatever its mechanism.

§ Further support of this conclusion has been obtained in unpublished experiments of Dr. I. B. Weinstein. *E. coli* W160-37 type 1–3 was depleted of arginine, treated with chloramphenicol (200μg/ml) to prevent protein synthesis, and incubated for 5 min with C^{14}-arginine (10 μg/ml) in the absence and in the presence of an excess of unlabeled ornithine (500 μg/ml). The results showed that ornithine did not interfere with the uptake of arginine.

[1] Vogel, H. J., and B. D. Davis, *Federation Proc.*, **11**, 485 (1952).

[2] Wijesundera, S., and D. D. Woods, *Biochem. Jour.*, **55**, viii (1953).

[3] Novick, A.. and L. Szilard, *Science*, **112**, 715 (1950); *Dynamics of Growth Processes* (Princeton: Princeton University Press, 1954), p. 21.

[4] Roberts, R. B., *et al.*, *Studies of Biosynthesis in Escherichia coli* (Carnegie Institution of Washington, D. C., 1955), p. 160.

[5] Gorini, L. (unpublished results).

[6] Gorini, L., and H. Kaufman, *Science*, **131**, 604 (1960).

[7] Davis, B. D., and E. S. Mingioli, *Jour. Bacteriol.*, **60**, 17 (1950).

[8] Gorini, L., *Bull. Soc. Chim. Biol.*, **40**, 1939 (1958).

[9] Gorini, L., and W. K. Maas, *Biochim. et Biophys. Acta*, **25**, 208 (1957).

[10] Pardee, A., F. Jacob, and J. Monod, *Jour. Mol. Biol.*, **1**, 165 (1959).

THE REPLICATION OF DESOXYRIBONUCLEIC ACID IN HEPATOCYTES

BY W. B. LOONEY*,†

DEPARTMENT OF RADIOTHERAPEUTICS, UNIVERSITY OF CAMBRIDGE, ENGLAND

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The process of replication of DNA in hepatocytes in rats following partial hepatectomy has been extensively studied by biochemical methods;[1–9] however, one of the major limitations of the biochemical methods is that it has not been possible to determine the time sequence of replication of the DNA in individual hepatocytes because of the changing population of hepatocytes synthesizing DNA.

Individual hepatocytes have been followed throughout replication of the DNA content by labeling nuclei with tritiated thymidine and measuring the DNA content microspectrophotometrically at varying times after administration. Quantitative autoradiography and microspectrophotometry have given independent methods for the measurement of the relative changes of DNA during replication in the same nuclei.[10, 14, 20]

Materials and Methods.—Combined autoradiographic and cytochemical studies have been carried out on rats sacrificed between $18^1/_2$ and 26 hr after partial hepatectomy. The weights have been between 200–300 grams and the age between 4–5 months. All rats were given intravenously 50 μc of tritiated thymidine (either 1.9 or 3 curies per millimol) diluted to a volume of 0.5 cc in normal saline.

The regenerating livers were minced with scissors and mixed. Random specimens of these small pieces of liver were taken and gently pressed between cover slips, followed by immersion in liquid propane. The specimens were quickly transferred to ethyl alcohol (cooled to -78 degrees C by solid CO_2).[11] All liver speci-

mens were stained by the Feulgen technique prior to preparation of the autoradiographs.[12] Autoradiographs of the liver specimens following Feulgen staining were prepared by the stripping film technique devised by Pelc.[13]

The measurement of the DNA content of the Feulgen stained nuclei has been made by means of two wavelength microspectrophotometry.[15, 16] The Schwarzchild–Villiger Phenomenon was not considered to introduce a significant error in these microspectrophotometric measurements.[17] The measurements were expressed in relative units between the 4N and 8N amount of DNA. The ratios of either the 2N to 4N, 4N to 8N, or 2N to 8N amount of DNA were within the limits of experimental error of the DNA determinations of either the 2N, 4N, or 8N hepatic nuclei.

The procedure recommended by Mendelsohn[18, 19] has been used routinely in the measurement of the DNA content of hepatocytes measured microspectrophotometrically. The total quantity of chromophore in the nucleus was obtained by substitution of these results into the appropriate equations and tables given by Patau.[16]

The combined autoradiographic and cytochemical measurements on the same nucleus were made in the following manner. All nuclei, both labeled and unlabeled, were mapped within a field. Grains over the labeled nuclei were counted and consecutive fields were mapped until a total of approximately 25 labeled nuclei had been recorded. The silver bromide grains were removed chemically and the DNA content measured microspectrophotometrically.

Results.—The results of the experiment in which tritiated thymidine was injected shortly after the onset of synthesis ($15^1/_2$ hr) are shown in Figure 1. During the availability of tritiated thymidine from $15^1/_2$ to $16^1/_2$ hr, only 1.51 ± 1.02 per cent of the hepatocytes were found to be labeled. The animal was sacrificed at $18^1/_2$ hr and the DNA content of 25 nuclei determined. The mean DNA content of the labeled hepatocytes was 34 per cent above the 4N value for DNA at this time (Table 1).

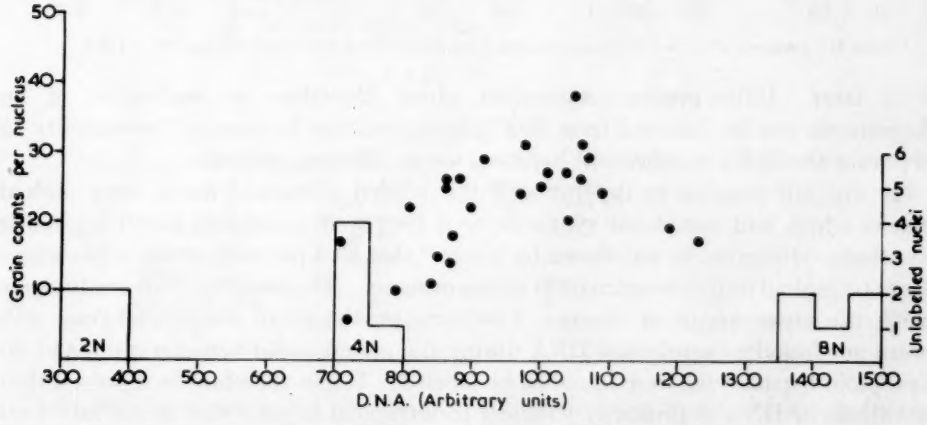

FIG. 1.—Plot of grain counts versus DNA content of the same hepatic nuclei of a rat given tritiated thymidine $15^1/_2$ hr after hepatectomy and sacrificed 3 hr later. ● labelled nuclei. □ unlabelled.

The hepatocytes in the experiment in which the tritiated thymidine was given at 17 hr, and the animal sacrificed at 23 hr, after hepatectomy is shown in Figure 1 (on page 700 of these PROCEEDINGS). Twenty-two of the 27 nuclei were

considered to be tetraploid nuclei which had doubled their DNA content to become octoploid nuclei. These results also demonstrated that 8 hr is an upper limit for the mean doubling for an hepatocyte.

Discussion.—A. Per cent of tritiated thymidine-labeled hepatocytes at different times after hepatectomy: It has been previously shown that the per cent of hepatocytes labeled with tritiated thymidine gradually increases from the onset of DNA synthesis at 15 hr until 20 hr after hepatectomy.[14] No labeled hepatocytes were found in either a normal rat or 2 rats injected at 14 hr after hepatectomy. The increase in the labeled hepatocytes between 15 and 20 hr after hepatectomy is approximately 4 per cent per hr. Between 20 hr and the onset of mitosis at 24 hr after hepatectomy, there is a decrease of labeled hepatocytes. This indicates that the initial group of hepatocytes which begin DNA synthesis at 15 hr after hepatectomy completes synthesis between 20 to 23 hr after hepatectomy. This also indicates that DNA synthesis and mitotic division occur in cycles, an observation first made by Ponfick in 1889.

B. *The mean time for replication of the DNA content of hepatocytes:* There is a large body of indirect information concerning the time for replication of the DNA in the hepatocytes of regenerating liver. It is well established that DNA synthesis begins approximately 15 hr after hepatectomy and mitosis begins at approximately 24 hr after hepatectomy and reaches a peak approximately

TABLE 1

CHANGE IN DNA CONTENT WITH TIME AFTER HEPATECTOMY MEASURED MICROSPECTROPHOTOMETRICALLY IN TRITIATED THYMIDINE LABELED-HEPATOCYTES

	Time after hepatectomy (hr)	Unlabeled Nuclei 2N	4N	8N	Labeled Nuclei Mean Content 25 Nuclei	S.D.	D*
21.7.59 (A)	$15^1/_2$–$18^1/_2$ (C)	347	735	1447	975	±115	0.34
17.3.59 (B)	17 –20 (C)	265	636	1146	963	±229	0.64
21.7.59 (B)	17 –23 (C)	314	692	1223	1317	±82	1.20
24.4.59	20 –$20^1/_2$ (C)	184	389	725	641	+77	0.75

* Mean DNA content of labeled nuclei expressed as a fraction between the 4N and 8N amount of DNA.

4 hr later. Little precise information about the time of replication of an hepatocyte can be obtained from this indirect evidence because of the inability to separate the DNA synthesis in hepatocytes in different cycles.

It was not possible to determine if the labeled tetraploid nuclei were diploid nuclei which had completed synthesis or if they were tetraploid nuclei beginning synthesis However, it was shown by Naora[21] that 83.4 per cent of the hepatocytes were tetraploid in rats weighing 200 grams or more. The results of this study agree with the observations of Naora. Furthermore, the small nonparenchymal cells were not found to synthesize DNA during the period under consideration, and no octoploid hepatocytes were found to be labeled. It can therefore be assumed that synthesis of DNA is primarily confined to tetraploid hepatocytes in the rats used in this study.

The results of this and other experiments have shown that the DNA content is doubled prior to division. The following method has been discussed to express the mean DNA content of the synthesizing hepatocytes in terms of the fraction of the content between the 4N and 8N amount of DNA.

L = mean content of labeled hepatocytes
8N = DNA content of octoploid hepatocytes
4N = DNA content of tetraploid hepatocytes
D = DNA content of labeled hepatocytes expressed as fraction of the content between 4N and 8N

The following equation expresses the changes in mean DNA content of the labeled nuclei from 4N to 8N as a change from 0 to 1 in relative units:

$$D = \frac{L - 4N}{8N - 4N}.$$

The results of the combined autoradiographic and cytochemical studies, in which the DNA content is expressed in terms of the fraction of the DNA content between 4N and 8N, are given in Table 1.

Estimates have been made of the time required for the DNA content of an hepatocyte to be doubled from the results of these experiments. The estimates have been made in the following way:

D = DNA content of labeled nuclei expressed as a fraction between the 4N and 8N amount of DNA
S = time after the onset of DNA synthesis at 15 hr until the sacrifice of the animal
T = time in hr for doubling of the DNA content of an hepatocyte.

Therefore,

$$\frac{D}{S} = \frac{1}{T}; \quad T = \frac{S}{D}.$$

For an example, in the 17-to 20-hr experiment, see Table 2.

$$D = 0.64$$
$$S = 20 - 15 = 5 \text{ hr}$$
$$T = \frac{5}{0.64} = 7.4 \text{ hr}$$

TABLE 2

ESTIMATIONS FOR TIME FOR DOUBLING OF THE DNA CONTENT OF AN HEPATOCYTE

No.	Date	Time after hepatectomy (hr)	Time after onset of synthesis at 15 hr (hr)	D*	Estimated doubling time (hr)
1	21.7.59 (A)	$15^1/_2$–$18^1/_2$	3.5	0.34	10.3
2	17.3.59 (B)	17 –20	5	0.64	7.8
3	21.7.59 (B)	17 –23	8	1.20	8.0
4	24.4.59	20 –$20^1/_2$	5.5	0.75	7.4

* DNA content of labeled nuclei expressed as a fraction between the 4N and 8N content.

These estimates are based on the assumption that the mean change in DNA content for 25 nuclei is constant. This is specifically pointed out to differentiate it from the autoradiographic results of individual nuclei in which a changing rate of synthesis was found. It was shown from both the autoradiographic and the combined autoradiographic and cytological results that DNA synthesis in regenerating liver is not synchronized. The change in the mean DNA content of the hepa-

tocytes synthesizing DNA measured microspectrophotometrically is in essence the integration of the rate of synthesis over the period under consideration. By assuming that the change in the mean DNA content of the population is constant, a reasonably good approximation of the doubling time of DNA of an hepatocyte is obtained. This is borne out by the experimental results shown in Figure 3 of Part II. It should be noted that a straight line can almost be fitted to the experimentally determined value of the mean DNA content of labeled hepatocytes at the different times after hepatectomy. The estimates of the time for replication of the DNA of an hepatocyte from the data of different experiments is given in Table 2.

It would be expected that the estimate of the doubling time based on the results obtained at the beginning of synthesis would be high since the rate of synthesis is lower at this time than at later times when the rate of synthesis has increased. Estimates based on the $15^1/_2$ to $18^1/_2$ hr experiment gave a doubling time of 10.3 hr.

The hepatocytes in the 17 to 23 hr experiment have doubled their DNA content. This gives an upper limit of DNA doubling time of 8 hr which is based directly on experimental results. Estimates based on both the 17 to 20 hr and the 20 to $20^1/_2$ hr experiments are in close agreement with the doubling time based directly on the experimental results above. The estimated doubling time for the labeled hepatocytes of these two experiments was 7.8 and 7.4 hr respectively.

It was shown that by 20 hr in the combined autoradiographic and cytochemical study that some of the hepatocytes had completed synthesis. The histogram of the grain counts per nucleus in the 20 to $20^1/_2$ hr experiment show therefore nuclei in all stages of synthesis. The shape of the histogram should also reflect the duration of the changing rates of synthesis of an hepatocyte in terms of the fraction of time necessary for DNA doubling. Since the histogram of the log of the grain counts per nucleus can be fitted to a normal curve, a normal curve with the same mean and standard deviation as the mean and standard deviation for the log of the grain counts per nucleus has been used as the model for the changing rates of DNA synthesis.

The general equation for the normal distribution is as follows: The frequency in any infinitesimally small range, dx, can be expressed as

$$df = \frac{1}{\sigma\sqrt{2\pi}}\, e^{-1/2 \cdot \frac{(x-\mu)^2}{\sigma^2}}\, dx$$

μ = mean of the distribution
σ = standard deviation
x = distance of the observation from the mean.

The following information about the log of the grain counts per nuclei for the control of the 20 to $20^1/_2$ hr experiment

μ_1 = the mean of the log of the grain counts per nucleus = 1.767
σ_1 = ± 0.225.

Substitution of the above information into the general equation for the normal distribution gives the following differential equation:

$$df = \frac{1}{\pm 0.225\sqrt{2\pi}} e^{-1/2} \frac{(x - 1.767)^2}{(0.225)^2} dx.$$

Since the histogram of the log of grain counts per nucleus can be fitted to a normal curve, the same curve can be used as the model for the changing rates of DNA synthesis. The time axis is superimposed on that of log grain counts, the 8 hr for replication covering that part of the axis within which readings fall 95 per cent of the time.

Transforming these values to the time scale, t = time in hours of the observation from the midpoint of the time for replication (8 hr).

The new standard deviation σ_2 is obtained from the knowledge that $\pm 1.96 \times$ S.D. will give values in which readings will fall 95 per cent of the time.

Therefore,

$$1.96 \times \sigma_2 = 4$$
$$\sigma_2 = 2.041.$$

By substitution into the general equation for the normal curve, the rate of DNA synthesis becomes

$$dR = \frac{1}{2.041\sqrt{2\pi}} e^{-1/2} \left(\frac{t}{2.041}\right)^2 dt,$$

where

t = time in hr
R = rate of DNA synthesis
σ_2 = 2.041 = new standard deviation
μ_2 = mean = 0.

A model for the changing rate of synthesis during the doubling of the DNA content of an hepatocyte is shown in Figure 2. This model was constructed by

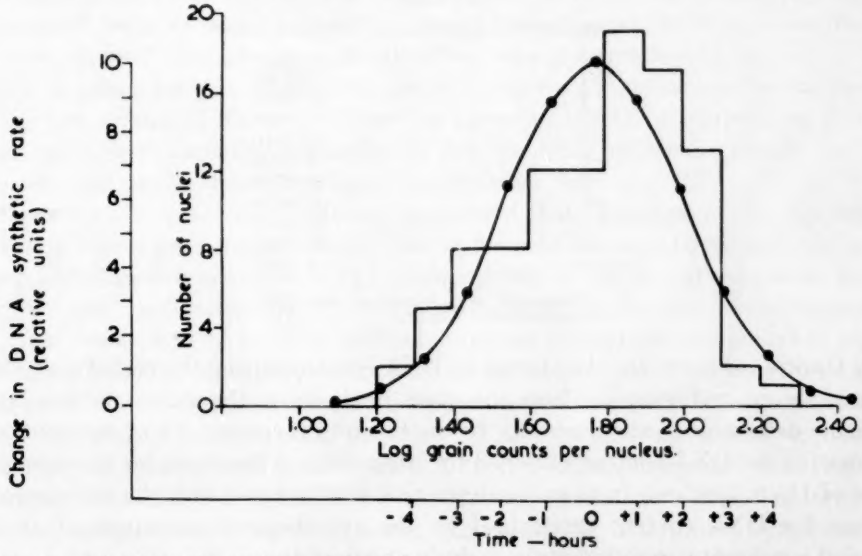

FIG. 2.—Model for the doubling of the DNA content of an hepatocyte (from histogram of log of grain count distribution and superimposed normal curve).

superimposing a normal curve with the same mean and standard deviation of the log of the grain counts per nucleus of the 20 to $20^1/_2$ hr experiment. Upon integration of the equation for the rate of DNA synthesis of an hepatocyte, the DNA content at time "t" is as follows:

$$\text{DNA (content)} = \int_{-4}^{t} \frac{1}{2.04\sqrt{2\pi}} e^{-1/2\left(\frac{t}{2.04}\right)^2} dt.$$

The values for the DNA content were obtained in the following manner. The results obtained experimentally from the autoradiographic studies have been used to determine the theoretical DNA content at time "t" during the period of replication:

$$t = \frac{x - \mu_1}{c},$$

where c is a conversion factor between the standard deviation for the log of the grain counts per nucleus σ_1 and the new standard deviation σ_2.

$$c = \frac{\sigma_1}{\sigma_2} = \frac{0.225}{2.041} = 0.110.$$

Therefore,

$$t = \frac{x - 1.767}{0.110}.$$

Upon substitution of these values into the preceding equation for DNA content and integrating, the following theoretical values of the DNA content have been obtained for different times during the 8-hr period of replication.

TABLE 3

THEORETICAL VALUES FOR DNA CONTENT

Time (hr)	Theoretical DNA Content*
−4	0.0250
−3	0.0683
−2	0.1610
−1	0.3096
0	0.4750
+1	0.6854
+2	0.8340
+3	0.9266
+4	0.9500

* Expressed as a fraction of the DNA content between 4N and 8N.

The theoretical curve for the change in DNA content during the period of replication is shown in Figure 3. Note the close proximity of the values for the DNA content determined experimentally to the theoretical curve. Thus, the predicted values for the DNA content obtained by integration of the curve for the changing rate of DNA synthesis from autoradiographic results agree with the experimental values for DNA content determined by the cytochemical measurement of the labeled hepatocytes at different times during replication.

The doubling time for the first 4 experiments was estimated based on the assump-

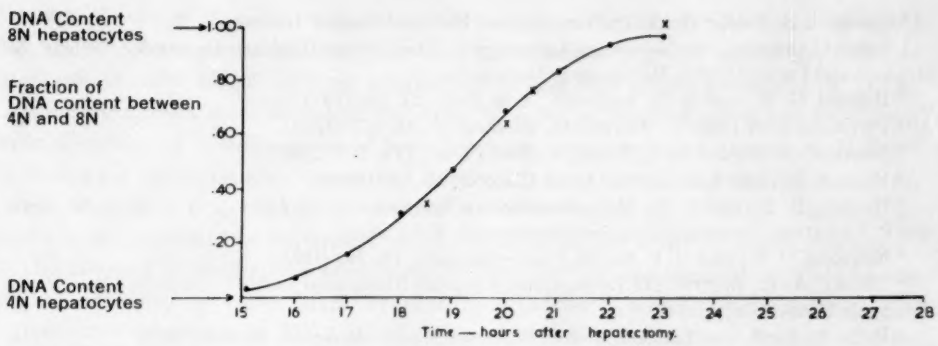

FIG. 3.—Change in DNA content with time after hepatectomy measured microspectrophotometrically in tritiated thymidine-labelled hepatocytes. ●—● theoretical curve for change in DNA content between 4N and 8N. × DNA content determined experimentally.

TABLE 4

ESTIMATIONS OF DNA DOUBLING BASED ON AN ASSUMED EXPONENTIAL CHANGE IN DNA CONTENT DURING REPLICATION

No.	Time after hepatectomy	Estimated DNA Content* Assuming 8 hr doubling time	Estimated DNA Content* Assuming 9 hr doubling time
1	$15^1/_2$–$18^1/_2$	1.12	0.91
2	17 –20	1.08	0.88
3	17 –23	1.00†	...
4	20 –$20^1/_2$	0.99	0.85

* DNA content of labeled nuclei expressed as a fraction between 4N and 8N amount.
† This value was determined experimentally.

tion that the change in DNA content during replication was exponential rather than linear.

The estimates of the DNA content at 8 hr obtained from substituting the values of the DNA content into the equation for the normal curve given previously and integrating are shown in Table 4. Note that all of the values are close to the anticipated value of one. In order to check further the reliability of these results it was assumed that the time for doubling was 9 hr rather than 8 hr. The values obtained by substitution into the equation for the normal curve are shown in the last column of Table 4. Since the values of the DNA content are all less than one it is evident that assuming an 8 hr doubling time more closely fits the experimental results than assuming a 9-hr doubling time.

Summary and Conclusions.—It has been found that the histogram for the log of the grain counts per hepatic nucleus could be fitted to a normal curve with the same mean and standard deviation. The changing rate of DNA synthesis of an hepatocyte during replication has therefore been expressed as an exponential function.

It has been possible to follow individual nuclei throughout the period of replication by labeling the nuclei with tritiated thymidine and measuring the DNA content of the labeled hepatocytes microspectrophotometrically at different times after tritiated thymidine administration. The experimental results from the cytochemical studies have been fitted to a curve from an integrated equation for changing rates of DNA synthesis. Thus, the theoretical curve predicted from the autoradiographic results agree with the experimentally determined cytochemical results. The mean time for replication of the DNA content of an hepatocyte was found to be 8 hr.

* Special U.S. Public Health Service Fellow, National Cancer Institute.

† Present address: Radiobiology Laboratory, The Johns Hopkins University, School of Hygiene and Public Health, Baltimore 5, Maryland.

[1] Higgins, G. M., and R. M. Anderson, *Arch. Path.*, **12**, 186 (1931).

[2] Davidson, J. N., and C. Waymouth, *Biochem. J.*, **38**, 375 (1944).

[3] Novikoff, A. B., and V. R. Potter, *J. Biol. Chem.*, **173**, 223 (1948).

[4] Price, J. M., and A. K. Laird, *Cancer Research*, **10**, 650 (1950).

[5] Holmes, B. E., and L. K. Mee, *Radiobiology Symposium*, 220, Leige (1954), ed. A. M. Bacq and P. Alexander (London: Butterworth Scientific Publ., 1955).

[6] Nygaard, O. F., and H. P. Rusch, *Cancer Research*, **15**, 240 (1955).

[7] Mirsky, A. E., in *Proc. III International Congress Biochemistry*, ed. C. Lie-Bacq (New York: Academic Press, 1956), p. 349.

[8] Beltz, R. E., J. Van Lancker, and V. R. Potter, *Cancer Research*, **17**, 688 (1957).

[9] Davidson, J. N., "Chemistry of the Liver Cell," *Brit. med. Bull.*, **13**, 77 (1957).

[10] Looney, W. B., Dissertation for Ph.D. Degree, University of Cambridge, England, November, 1959.

[11] Simpson, W. L., *Anat. Rec.*, **80**, 173 (1941).

[12] Lillie, R. D., *Histopathologic Technic and Practical Histochemistry* (New York: Blakiston, 1954).

[13] Pelc, S. R., "Autoradiographic Technique," *Nature*, **160**, 749 (1947).

[14] Looney, W. B., *Proceedings of the IXth International Radiological Congress* (Munich: Georg Thieme, Verlag (in press).

[15] Ornstein, L., *Lab. Invest.*, **1**, 250 (1952).

[16] Patau, K., *Chromosoma*, **5**, 341 (1952–53).

[17] Naora, H., *Science*, **114**, 279 (1951)

[18] Mendelsohn, M. L, Dissertation for Ph.D. Degree, University of Cambridge, Cambridge, England, 1957.

[19] Mendelsohn, M. L., *J. Biophys. Biochem. Cytol*, **4**, 407 (1958).

[20] Looney, W. B., in *Faraday Society Conference on the Cell Nucleus*, Ed. B. E. Holmes (London: Butterworth Scientific Publ., 1960).

[21] Naora, H., *J. Biophy. Biochem. Cytol.*, **3**, 949 (1957).

THE EFFECT OF IRRADIATION ON THE REPLICATION OF DESOXYRIBONUCLEIC ACID IN HEPATOCYTES

BY W. B. LOONEY,* R. C. CAMPBELL,† AND BARBARA E. HOLMES

DEPARTMENT OF RADIOTHERAPEUTICS, UNIVERSITY OF CAMBRIDGE, ENGLAND

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By 1925 it was generally accepted that the cell nucleus was more sensitive to the effects of irradiation than the cytoplasm.[1] The discovery by Muller[2] in 1927 that X rays could produce gene mutations firmly established the importance of the effects of irradiation on the genetic material in the nucleus. Mitchell[3, 4] and Euler and Hevesy[5] were first to show that irradiation would produce disturbances in nucleic acid metabolism.

Additional results suggesting that irradiation directly affects DNA synthesis were reported by Holmes,[6] Hevesy,[7] Skipper and Mitchell,[8] Pelc and Howard,[9] Klein and Forssberg,[10] Vermund, Barnum, Huseby, and Stenstrom,[11] Bennett, Kelly, and Kreukel,[12] Lajtha, Oliver, and Ellis,[13] Looney,[14, 15] Lajtha, Kumatori, Oliver, and Ellis,[16] Holmes and Mee,[17] Beltz, Van Lancker, and Potter,[18] and

Stocken.[19] Some of the more pertinent findings were that (1) DNA was synthesized during interphase in the mitotic cycle and (2) irradiation given before the onset of synthesis is more effective than irradiation during the active synthesis of DNA. Conflicting results were found in the large number of studies carried out under a wide diversity of conditions on different organs, systems, and animals. Kelly,[20] in reviewing the extensive amount of information on the effects of irradiation on DNA, suggested that almost all the results were consistent with the assumption that the results could be explained by factors such as cell death or mitotic delay.

The labeling of the hepatocytes immediately after irradiation and sacrifice of the animals at varying times after irradiation provides a means of identifying the hepatocytes synthesizing DNA at the time of irradiation. Measurement of the DNA content of the labeled nuclei microspectrophotometrically permits the determination of the changes in the DNA content of individual nuclei following irradiation.

Materials and Methods.—Combined autoradiographic and cytochemical studies have been carried out on irradiated and paired control rats sacrificed between 17 and 26½ hr after partial hepatectomy. X radiation was given at the rate of 127 r per min for a total dose of 3,000 r. Tritiated thymidine was given immediately after the termination of irradiation. The remaining lobe of the liver was directly irradiated and the remainder of the body shielded. (See *Materials and Methods*, preceding paper, this issue.)

Results.—The results of the combined cytochemical and autoradiographic measurements on the same nucleus are illustrated by an experiment in which tritiated thymidine and 3,000 r of X irradiation were given at 17 hr after hepatectomy and both irradiated and paired control animals sacrificed 6 hr later. (See Fig. 1.) All of the hepatocytes in the paired control which were synthesizing DNA at 17 hr after hepatectomy had doubled their DNA content by 23 hr after hepatectomy. However, the mean DNA content of the labeled hepatocytes in the irradiated animal was only 63 per cent above the 4N value for DNA (see Table 1).

In one experiment the tritiated thymidine was injected at 17 hr after hepatectomy and the animal sacrificed 9 hr later (Fig. 2). It was found in the control animal that 14 of the hepatocytes labeled with tritiated thymidine at 17 hr after

TABLE 1

CHANGE IN DNA CONTENT WITH TIME AFTER HEPATECTOMY MEASURED MICROSPECTROPHOTOMETRICALLY IN TRITIATED THYMIDINE-LABELED HEPATOCYTES

			Unlabeled Nuclei			Labeled Nuclei			
Date	Time after hepatectomy (hr)		2 N	4 N	8 N	Mean content 25 nuclei	S.D.	D*	Mean† grain counts per nucleus (25 nuclei)
21.7.59 (A)	15½–18½	(C)	347	735	1447	975	±115	0.34	23.74
		(R)	364	714	1382	915	± 52	0.27	11.00
21.7.59 (B)	17–23	(C)	314	692	1223	1317	± 82	1.20	33.78
		(R)	377	801	1575	1291	±173	0.63	24.33
17.3.59 (A)	17–26	(C)	222	554	935	904	±119	0.92	19.58
		(R)	138	322	662	640	± 75	0.94	14.89
24.4.59	20–20½	(C)	184	389	725	641	± 77	0.75	52.75
		(R)	209	440	790	582	± 77	0.41	15.35

* Mean DNA content of labeled nuclei expressed as a fraction between the 4N and 8N amount of DNA.
† These grain counts were made on the labeled hepatocytes before removal and measurement of the DNA content.
(C) control. (R) irradiated.

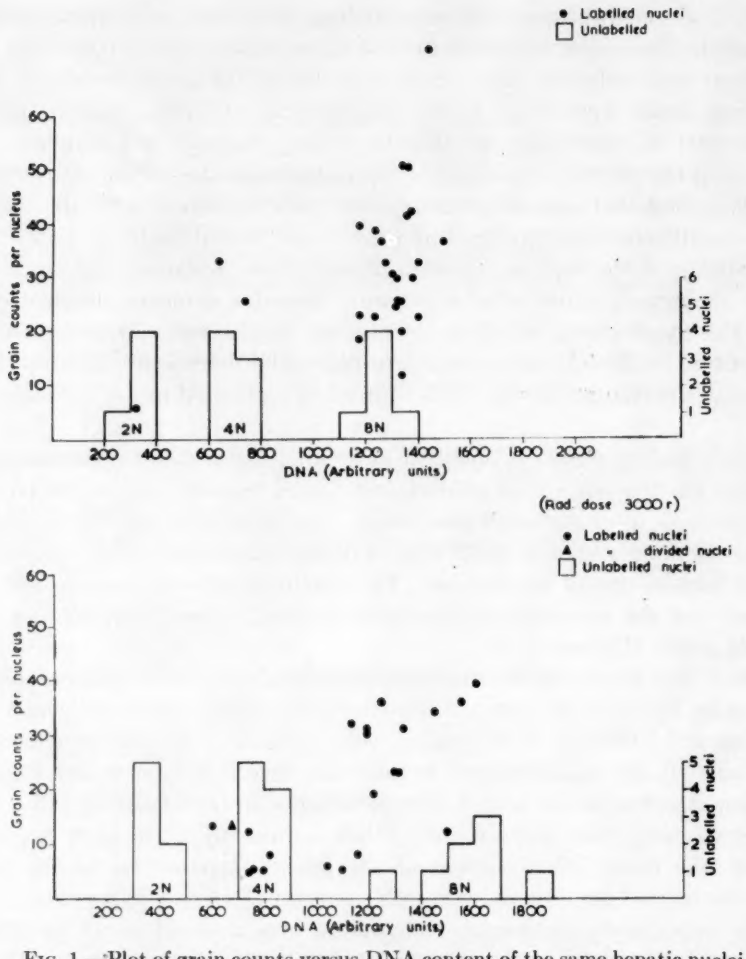

FIG. 1.—Plot of grain counts versus DNA content of the same hepatic nuclei of a rat given tritiated thymidine at 17 hr after hepatectomy and sacrificed 6 hr later.

hepatectomy had completed the doubling of the DNA content and divided. At this time the mean DNA content of the labeled hepatocytes of the irradiated animal was 92 per cent above the 4N amount of DNA.

Estimates have been made for the time necessary for DNA replication in the labeled hepatocyte following 3,000 r of irradiation. The estimates of the time for replication of the DNA of an hepatocyte from the data of different experiments following irradiation are given in Table 2. A straight line can almost be fitted to the experimentally determined value of the mean DNA content of labeled hepatocytes of both irradiated and control animals (Fig. 3).

In one experiment tritiated thymidine was injected at 20 hr after hepatectomy and the animal sacrificed 30 min after injection. As tritiated thymidine is only available for a short time, the animal was sacrificed shortly after administration to determine what correlation, if any, could be made between the grain counts per nucleus in the irradiated and control nuclei and the DNA content in the irradiated

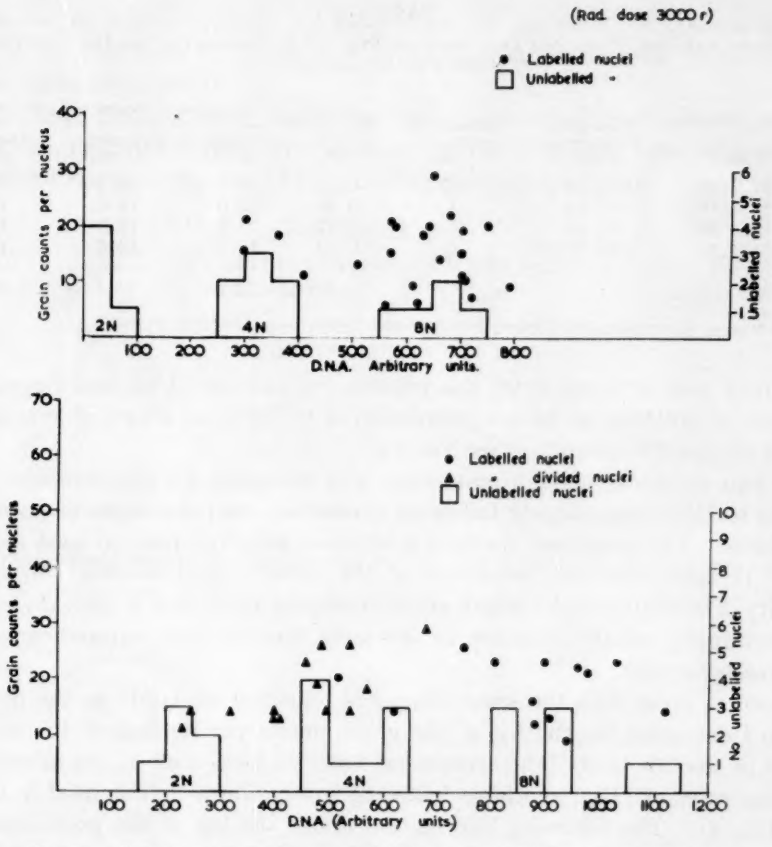

Fig. 2.—Plot of grain counts versus DNA content of the same hepatic nuclei of a rat given tritiated thymidine at 17 hr after hepatectomy and sacrificed 9 hr later.

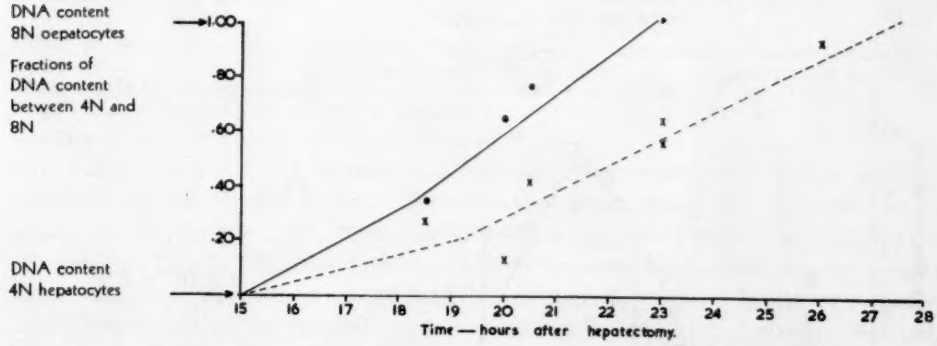

FIG. 3.—.Change in DNA content with time after hepatectomy measured microspectrophotometrically in tritiated thymidine labeled hepatocytes. Key: • control. × • irradiated μ.

and control nuclei. Twenty hr after hepatectomy was selected because it is the time of most active DNA synthesis as shown by tritiated thymidine and autoradiographic means. Twenty hr was also selected because little change in the

TABLE 2

ESTIMATIONS FOR THE TIME FOR DOUBLING OF THE DNA CONTENT OF AN HEPATOCYTE AFTER 3,000 R OF IRRADIATION

No.	Date	Time after Hepatectomy (hr)	Duration of synthesis (hr)	D*	Estimated Doubling Time (hr)		
					Linear model	Exponential model	Quadratic model
1	21.7.59	$15^1/_2$–$18^1/_2$	3.5	0.27	13.0	9.5	10.3
2	21.7.59	17–23	8	0.63	12.6	14.0	13.6
3	17.3.59	17–26	11	0.92	13.7	14.0	13.3
4	24.4.59	20–$20^1/_2$	5.5	0.41	12.4	12.8	12.5
				Mean	12.9	12.4	12.4

* DNA content of labeled nuclei expressed as a fraction between the 4 N and 8 N content.

per cent of labeled hepatocytes was present, yet sufficient time had elapsed since the onset of synthesis to have a population of nuclei in all stages of synthesis between a 4N and 8N amount. (See Table 1.)

The two methods compare reasonably well as means for the determination of changes in DNA immediately following irradiation with the limits of error of the procedures. The combined method is superior to either method used independently. It eliminates the weakness of the cytochemical method which is the difficulty of detecting nuclei which are synthesizing DNA and it provides a means for determining relative changes in the same nucleus both autoradiographically and cytochemically.

A normal curve with the same mean and standard deviation as the mean and standard deviation for the log of the grain counts per nucleus of the irradiated animal in the 20- to $20^1/_2$-hr experiment has also been used as the model for the changing rates of DNA synthesis following irradiation as it was used in the control (Fig. 4). The following information about the log of the grain counts per nucleus for the irradiated animal of the 20- to $20^1/_2$-hr experiment, has been substituted into the general equation for the normal distribution.

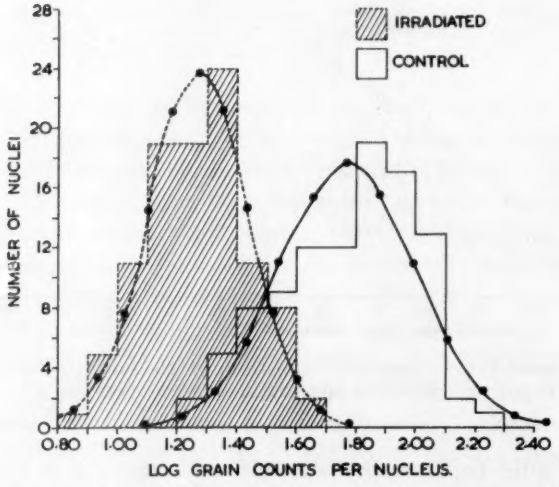

Fig. 4.—Histogram of log of grain count distribution of nuclei with superimposed normal curve.

Mean log of grain counts = $\mu_1 = 1.266.$

Standard deviation = $\sigma_1 = \pm 0.167.$

Thus,

$$df = \frac{1}{\pm 0.167\sqrt{2\pi}} e^{-\frac{1}{2}\cdot\frac{(x-1.2659)^2}{(0.167^2)}} dx.$$

The time axis is superimposed on that of log grain counts, the 13 hr for replication covering that part of

the axis within which readings fall 95 per cent of the time. Transforming these values to the time scale, t = time in hr of the observation from the midpoint of the time for replication (13 hr).

Upon substitution of these values into the equation for DNA content and integrating, the following theoretical values of the DNA content have been obtained for different times during the 13-hr period of replication.

TABLE 3

THEORETICAL VALUES FOR DNA CONTENT

Time (hr)	Theoretical DNA Content*	Time (hr)	Theoretical DNA Content
−6.5	—	+1.0	0.5935
−6.0	0.0101	+2.0	0.7018
−5.0	0.0409	+3.0	0.7921
−4.0	0.0889	+4.0	0.8611
−3.0	0.1579	+5.0	0.9091
−2.0	0.2482	+6.0	0.9398
−1.0	0.3465	+6.5	0.9500
0	0.4750	...	...

* Expressed as a fraction of the DNA content between 4N and 8N.

The theoretical curve for the change in the DNA content of hepatocytes, after 3,000 r of irradiation, and during the process of replication is shown in Figure 5.

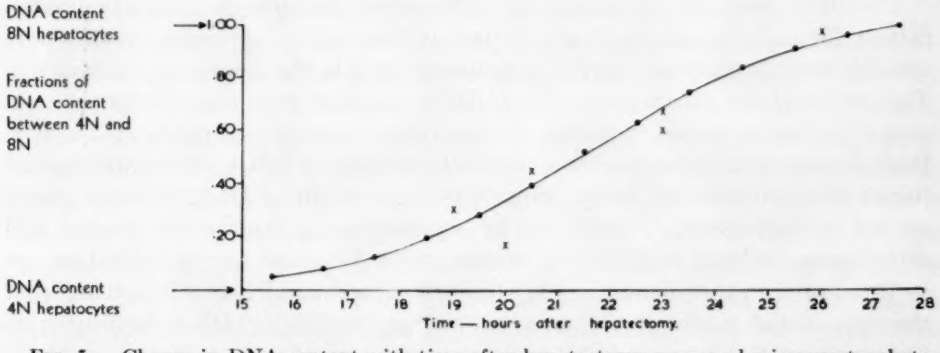

FIG. 5.—Change in DNA content with time after hepatectomy measured microspectrophotometrically in tritiated thymidine labeled hepatocytes. Key: ·——·, theoretical curve for change in DNA content between 4N and 8N after 3000 r of irradiation. ×, DNA content determined experimentally after 3000 r of irradiation.

The values for the DNA content determined experimentally are not in as close proximity as the control values. However, the agreement is still considered to be good. Thus, the predicted values for the DNA content obtained by integration of the curve for the changing rate of DNA synthesis from autoradiographic results agree with the experimental values for DNA content determined by the cytochemical measurement of the labeled hepatocytes at different times during replication. Both the autoradiographic and cytochemical results suggest that irradiation primarily causes a reduction in the DNA synthetic rate.

Estimates of the DNA doubling time have been made by using the exponential equation for changing rates of synthesis rather than the linear equation as used initially. This has been done by assuming different times for doubling of the DNA and determining the DNA content at the end of the assumed time. Estimates

of the DNA content at the end of 12 hr, 12.6 hr, and 13 hr have been made from the results of the DNA content found at different times in the four experiments given in Table 4. Substitution of a doubling time of 12.6 hr into the exponential equation for the change in DNA content during replication most closely approximates the doubled amount of DNA.

TABLE 4

ESTIMATIONS OF DNA DOUBLING BASED ON AN ASSUMED EXPONENTIAL CHANGE IN DNA CONTENT DURING REPLICATION

No.	Time after Hepatectomy (hr)	Estimated DNA Content* Based on an Assumed Doubling Time of: (a) 12 hr	(b) 12.6 hr	(c) 13 hr
1	$15^1/_2$–$18^1/_2$	0.67	0.63	0.59
2	17–23	1.14	1.08	1.03
3	17–26	1.00	0.98	0.97
4	20–$20^1/_2$	1.00	0.93	0.88

* DNA content expressed as a fraction between the 4N and 8N content.

Discussion.—It is evident from these investigations that any attempt to evolve a simple working model illustrating how irradiation affects DNA synthesis is fraught with many inherent dangers. However, the results of other investigations and the results of this study do permit certain inferences to be made in regard to the sequence of events resulting in disturbance in DNA synthesis by irradiation.

The linear model for the change in DNA content during replication was unsatisfactory for both the irradiated and control experiments. Two curved models were used to directly estimate the DNA doubling times in the irradiated experiments. The results of the direct estimation of DNA doubling time using directly an exponential and a simple quadratic (a parabola) equation are given in Table 2. Both the exponential and quadratic models fit reasonably well with the experimental results obtained cytochemically. Since the changing rate of DNA synthesis, based on the autoradiographic results, can be expressed as an exponential function and since the exponential model for the change in DNA content during replication can be fitted to the experimental results obtained cytochemically, it is considered that the exponential model is the most satisfactory model for DNA replication in hepatocytes.

One of the well-established examples of the direct effect of irradiation is the production of mutations in Drosophila. Analysis of the results of this and other investigations by the single-hit target theory has provided much useful information about the quantitative relationship between irradiation and gene mutations. The use of this theory has also been effectively utilized in the study of inactivation of enzymes, phages, and viruses. The studies on the quantitative relationship between irradiation and molecular inactivation have been reviewed by Guild.[21] The correlation between the radiation inactivation and estimates of molecular weights based on target size and the known molecular weight for 32 molecules with molecular weights ranging from 3×10^2 to 10^7 was presented. The correlation between these results and the known molecular weights is within a factor of 2 or less, made under standardized conditions. Guild states that this empiric relationship seems to demonstrate that a primary ionizing event anywhere in a biological molecule has a probability of near unity of inactivating as its special function.

Puck, Morkovin, Marcus, and Cieciura[22] reported that the mean lethal dose for a

large number of normal and malignant human cells grown in tissue culture was between 50 to 150 r. It was found that the survival curve for the more radioresistant cells approximated a 2-hit type of curve. The survival curves for the more radiosensitive cells such as fibroblasts could be fitted by either a 1- or 2-hit curve. They conclude that the radiosensitivity of these cells could be explained on the basis of a defect resulting from primary damage localized in one or more chromosomes.

Alexander and Charlesby[23] consider that the accumulation of information of the effects of irradiation in polymers has demonstrated that the difference between direct and indirect effects is much less than previously thought. Mitchell[24] suggests, on general grounds, that the indirect effects from the diffusion of effective agents to the essential target do not represent the most important factor in the production of biological effects of irradiation.

The results of recent investigations have given a more precise picture of the time sequence of the pre-synthetic period and the effects of irradiation on the precursors of DNA in the pre-synthetic period. An increase in the desoxyribosidic compounds has been found in the pre-synthetic period of DNA in rats following hepatectomy by both Schneider and Brownell[25] and Jaffe, Lajtha, Lascelles, Ord, and Stocken.[26] The fact that thymidylate kinase is indetectable in normal rat liver had led Bollum, Anderegg, McElya, and Potter[27] to suggest that the induction of this enzyme is essential for the build-up of deoxynucleotides.

The results of Jaffe *et al.* show that an increase in the desoxynucleoside compounds occurs after irradiation and they suggest that the irradiation is affecting the process by which the desoxynucleosides are converted to deoxynucleotides. These investigations suggest, therefore, that the low level of irradiation given in these experiments affects the process by which thymidylic kinase is induced, rather than inhibition of the existing enzyme. Since the presence of a substrate is involved in the evocation of an induced enzyme, and since at 8 to 12 hr after hepatectomy cytidine deoxynucleoside is the predominant deoxy-compound, they suggest that a nucleoprotein complex may be the synthetic site. They suggest further that the disturbances found after irradiation may be due to a derangement of nucleoprotein at the site of synthesis of the enzyme.

Bloch and Godman[28] have shown from cytochemical studies that the synthesis of histones occurred at the same time as the synthesis of DNA in cells preparing for division. Holmes[29] found that nuclear "residue" protein synthesis paralleled DNA synthesis in regenerating rat liver as determined by isotopic incorporation of labeled C-14 lysine and C-14 arginine. The incorporation of these basic amino acids into the residual proteins indicates that the formation of the basic proteins closely parallels DNA synthesis. Their formation may occur simultaneously with DNA synthesis and may be an essential part of the replication process of the nucleus.

Numerous studies have shown that the nucleoproteins are radiosensitive and may be more affected by irradiation than DNA. Anderson and Fisher[30] found that viscosity reductions can be obtained in solutions of DNAP with as little as 25 r. They emphasize the fact that kilo-roentgen doses of irradiation are necessary to produce changes in viscosity in pure DNA in solution.

The preceding results and the results of this investigation would be consistent with the hypothesis of Mitchell[31] that the primary effect of irradiation is the pro-

duction of a macromolecular lesion which involves the desoxyribonucleoprotein system. A mechanism of separation analogous to the opening of a "zipper" is postulated to occur at some time during the duplication of the DNA or DNAP. Therefore, the changes produced by one ion pair occurring at any point in a sensitive region could block the process of duplication.

The results of this investigation have demonstrated that, in regenerating liver, the factor of change in the cell population does not enter into the consideration of the immediate effects of irradiation on DNA synthesis during the period under study. This was shown by two independent methods. Firstly, no significant difference was shown between the total number of cells in the livers of the irradiated and control animals. Secondly, no significant difference could be shown between the per cent of the hepatocytes labeled with tritiated thymidine in the irradiated control animals of 14 experiments carried out between 17 and $26^1/_2$ hr after hepatectomy.

The comparison of the cumulative distribution curve for the grain counts per nucleus of the irradiated with the paired control has given some of the more convincing results of this investigation with regard to the direct effects of irradiation on DNA synthesis. The reduction in the irradiated grain counts per nucleus was proportional to the control grain counts per nucleus over a range which varied by a factor of 10 or more. These results suggest that the reduction in the rate of DNA synthesis following irradiation is proportionate to the rate of synthesis at the time of irradiation (Looney[14]).

Tritiated thymidine was given at the end of the irradiation, and in 14 experiments the rate of uptake was 42.42 per cent of the paired control values. It would, therefore, appear that the reduction of the labeled thymidine incorporation immediately after irradiation is a measure of the direct effect of irradiation on the formation of the DNA molecule.

From previous calculations it was estimated that an average of 3,000 molecules were formed per min in the 8 hr necessary for replication in the controls. Therefore, $3{,}000 \times 0.42 = 1{,}260$ molecules per min = the rate of formation of DNA immediately after irradiation.

The estimate that 3 ion pairs within the DNA of an hepatocyte inhibits the replication of one DNA molecule suggests that an extremely small number of ionizing effects are needed to prevent the formation of a new molecule of DNA. It is of interest to compare the estimation of the ionizing effects necessary to prevent formation of a new DNA molecule with the estimate for the inactivation of a large number of molecules[21] and the 1- and 2-hit curves attributed to primary damage in one or more chromosomes.[22]

The sacrifice of the animals at different times after irradiation and tritiated thymidine administration has permitted the tracing of the labeled hepatocytes after irradiation until the DNA content is doubled. The determination of the DNA content cytochemically after irradiation is in all probability a measure of the effect of irradiation on the entire process of DNA replication.

The results of these combined autoradiographic and cytochemical experiments indicate that after 3,000 r of irradiation the mean synthetic time is increased from 8 hr to 13 hr. Therefore, $\frac{1.34 \times 10^6}{13 \times 60} = 1{,}720$ molecules per min = average rate of formation of DNA after irradiation. Therefore, 15 per cent of this reduction might

be considered to be directly affecting the formation of DNA molecules and the remainder of the reduction affecting the entire biosynthetic process by which DNA is produced.

Summary and Conclusions.—Autoradiographic studies with tritiated thymidine have shown that no significant difference exists between the number of labeled hepatocytes in the paired controls and the irradiated rats after partial hepatectomy and immediately following 3,000 r of X radiation. Quantitative autoradiographic studies with tritiated thymidine have given results which suggest that the reduction in DNA synthesis immediately following irradiation is directly proportional to the rate of synthesis at the time of irradiation.

The mean time for replication of the DNA content of an hepatocyte of 8 hr in the paired controls was increased to 13 hr in the irradiated animals. The shape of the exponential curve for the changing DNA content during the period of replication following irradiation was similar to the shape of the curve in the controls. The results of this investigation are, therefore, consistent with the assumption that irradiation directly affects the biosynthetic process of DNA replication.

We would like to express our sincere appreciation to Prof. Joseph S. Mitchell, F.R.S., for his assistance and encouragement throughout this investigation, and for making facilities available in his department for this study. We would also like to acknowledge the cooperation and assistance given us by the entire staff of the Department of Radiotherapeutics. Their willingness to discuss the various technical problems concerning this study has been of great benefit. It has been possible for one of us (W. B. L.) to carry out this study through a Special United States Public Health Service Fellowship of the National Cancer Institute.

* Present address: Radiobiology Laboratory, The Johns Hopkins University School of Hygiene and Public Health, Baltimore 5, Maryland.

† University Lecturer in Statistics, Department of Agriculture, University of Cambridge.

§ The average rate of synthesis of 3,000 molecules of DNA per minute was obtained from:

(1) Mw of DNA molecule = 6,000,000

(2) Time of replication = 8 hr

(3) Weight of DNA in an hepatocyte = 1.34×10^{-11} gm.

(4) Number of molecules 1.34×10^6

[1] Heilbrunn, L. V., and D. Mazia, *Biological Effects of Radiation*, ed. B. M. Duggar (New York: McGraw-Hill, 1936), chap. 18.

[2] Muller, H. J., *Science*, **66**, 84 (1927).

[3] Mitchell, J. S., *Nature*, **146**, 272 (1940).

[4] Mitchell, J. S., *Brit. J. Exp. Path.*, **23**, 285 (1942).

[5] Euler, H., and G. Hevesy, *K. Danske Vidensk. Selsk. Biol. Medd.*, **17**, 1 (1942).

[6] Holmes, B. E., *Brit. J. Radiol.*, **20**, 450 (1947).

[7] Hevesy, G., *Nature*, **163**, 869 (1949).

[8] Skipper, H. E. and J. H. Mitchell, *Cancer*, **4**, 363 (1951).

[9] Howard, A., and S. R. Pelc, *Heredity* (Suppl.) **6**, 261 (1953).

[10] Klein, G., and A. Forssberg, *Exptl. Cell Research*, **6**, 211 (1954).

[11] Vermund, H., C. P. Barnum, R. A. Huseby, and K. W. Stenstrom, *Cancer Research*, **13**, 633 (1953).

[12] Bennett, E. L., L. S. Kelly, and B. Kreukel, *Fed. Proc.*, **13**, 181 (1954).

[13] Lajtha, L. G., R. Oliver, and F. Ellis, *Radiobiology Symposium*, Liege, 1954, ed. A. M. Bacq and P. Alexander (London: Butterworth Scientific Publ., 1954), p. 216.

[14] Looney, W. B., *Proceedings of the IXth International Radiological Congress* (Munich: Georg Thieme Verlag, in press).

[15] Looney, W. B., *Faraday Society Conference on the Cell Nucleus*, Cambridge, England, Aug. 31–Sept. 1, 1959, ed. B. E. Holmes (London: Butterworth Scientific Publ., 1960).

[16] Lajtha, L. G., T. Kumatori, R. Oliver, and F. Ellis, *Radiation Research*, **8**, 1 (1958).

[17] Holmes. B. E., and L. K. Mee, *Radiobiology Symposium*, Liege, 1954, ed. Z. M. Bacq and P. Alexander, (London: Butterworth Scientific Publ., 1954), p. 220.

[18] Beltz, R. E., J. Van Lancker, and V. R. Potter, *Cancer Research*, **17**, 688 (1957).

[19] Stocken, L. A., *Radiation Research*, Suppl. I, 53 (1959).

[20] Kelly, L. S., *Progress in Biophysics and Biophysical Chemistry*, vol. 8 (London: Pergamon Press, 1957), p. 144.

[21] Guild, W. R., *Radiation Research*, **9**, 124 (1958).

[22] Puck, T. T., D. Morkovin, P. I. Marcus, and S. J. Cieciura, *J. Exp. Med.*, **106**, 485 (1957).

[23] Alexander, P. A., and A. Charlesby, *Nature*, **173**, 578 (1954).

[24] Mitchell, J. S., *Studies in Radiotherapeutics*, (Oxford: Blackwells, 1959).

[25] Schneider, W. B., and L. W. Brownell, *J. Nat. Cancer Inst.* **18**, 579 (1957).

[26] Jaffe, J. J., L. G. Lajtha, J. Lascelles, M. G. Ord, and L. A. Stocken, *International J. Radiation Biology I*, 241 (1959).

[27] Bollum, F. J., J. W. Anderegg, A. B. McElya, and V. R. Potter, *Cancer Research*, **20**, 138 (1960).

[28] Bloch, D. P., and G. C. Godman, *J. Biophys. Biochem. Cytol.*, **1**, 17 (1955).

[29] Holmes, B. E., *Abstract, Fourth International Biochemical Congress*, Vienna, 1958.

[30] Anderson, N. G., and W. D. Fisher, *Faraday Society Conference on the Cell Nucleus*, Aug. 31–Sept. 1, Cambridge, England, 1959 (London: Butterworth Scientific Publ., 1960).

[31] Mitchell, J. S., *J. Colloid Science*, **11**, 317 (1956).

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*GENE STRUCTURE AND FUNCTION IN NEUROSPORA**

BY R. P. WAGNER, CAROLYN E. SOMERS,† AND ARLOA BERGQUIST

THE GENETICS LABORATORY OF THE DEPARTMENT OF ZOOLOGY, THE UNIVERSITY OF TEXAS, AUSTIN

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The considerable effort made over the last decade and a half to analyze more incisively the structure and function of those segments of genetic material called genes has led both to the discovery of some remarkable relationships between closely linked segments of chromosomes, and a general obfuscation of what was previously a rather clear concept of genes.

The discovery of pseudoalleles in *Drosophila* by Oliver, Green, and Lewis,[1–3] which partially demolished the allele concept,[4] the studies of the fine structure of the genetic material in phage,[5] and the discovery and experimental exploitation of the phenonena of transformation[6] and transduction[7] in bacteria have made it evident that the event leading to recombination can occur probably anywhere along the length of the chromosome or genetic string inside or outside of "genes." Furthermore, much of this work, particularly that on pseudoalleles and transduction, has made it clear that closely linked genes, or functional segments, may have related or cooperative functions. This has been especially well illustrated in *Salmonella* by Demerec[8] and others.[9–11]

Carrying the cis-trans test of complementation versus non-complementation over into the protocaryotes such as phage and bacteria has resulted in the recog-

nition of a functional entity called a cistron by Benzer.[12] This may be considered a segment of genetic material within which complementation, i.e., the production of, or approach to wild type, will *not* occur if two such segments with "mutations" within their limits are present together in a heterozygous cell, or a heterocaryon. The existence of cistrons in phage, and the close proximity of apparently related cistrons, linearly arranged, has been demonstrated by Benzer.[5] This he has done by comparing the results of complementation studies in phage with recombination data obtained from matings. In a sense, the term cistron has assumed part of the meaning of the old term allele, but not all, because recombinations can occur within the cistron.

Complementation studies with eucaryotes such as fungi, notably *Neurospora*, made by forming heterocaryons between "allelic" nutritional mutants, and noting whether the heterocaryons can grow on minimal medium, have demonstrated that the cis-trans effect can be studied in them even though they are haploids. Thus, Catcheside, Fincham, and Giles and associates,[13-17] among others, have demonstrated that in certain argininеless, amino acidless, and adenineless mutants of *Neurospora crassa*, complementation with the production of growth in absence of the required compound may result in certain heterocaryons. The results of these workers have demonstrated that the old heterocaryon test for "allelism"[18] is no longer as meaningful as previously thought, for two mutants which do not show measurable recombination may complement. Furthermore, they have directly demonstrated that an active enzyme is synthesized by a heterocaryon between two mutants which cannot form the enzyme alone as homocaryons. Woodward[19] has recently carried the experiment one step further by demonstrating that extracts from homocaryons of two mutants capable of complementing in heterocaryons will form active enzyme *in vitro* on being mixed together.

The studies on recombination and complementation involving short segments of genetic material, exemplified by the work of those cited above, have made it abundantly clear not only that the gene locus is a complex entity, but also that certain interactions must occur between the products of presumably "allelic" loci which five years ago had been unsuspected or at least unverified. The precise relationship between positive interaction (as manifested by complementation and diagrammed in a complementation map) and the recombination map is uncertain. Benzer[5] has demonstrated a relationship in phage, and Case and Giles[17] have done so in *Neurospora*. But in both cases difficulty exists in that the primary gene function is either not known, as in phage, or is not yet analyzable at the enzymatic or protein level.

Presented in this communication is a preliminary account of the results of some experiments carried out with certain mutants of *Neurospora crassa* which require isoleucine and valine. The results answer no questions that have not been answered previously, but we believe that they indicate that we may here have a system which may help to answer some of the unanswered questions, notably those dealing with the relationship between complementation and recombination. It is our thesis that a clear understanding of what we mean by gene structure and function can only be arrived at by clarifying this relationship as one among many other related problems.

Origin and Destination of Mutant Strains.—All isoleucine-valine mutants with

the exception of 16117 were obtained by the application of the screening technique of Woodward *et al.*[22] after irradiation of the conidia of the parent stocks by ultraviolet light. Strains T304, T305, T318, T319, T320, T321, T322, T324, T325, T311, and T323 were derived from the wild type strain Emerson 5256A. Strains T331, T326, T327, T328, T329, T330, and T332 were derived from wild type T1956a which was originally started from an ascospore from a cross between the two wild type strains, Em5256A × Em5297a. Strain 16117 is an X-ray induced mutant[21] and was obtained from the California Institute of Technology.

In subsequent discussion the isoleucine-valine mutants obtained in this laboratory (T304 through T332 above) will be referred to collectively as the *T iv* mutants. In addition, the *T iv* mutants, when referred to specifically, will have the T omitted.

Growth of the Mutants.—None of the mutants, except 328, gives more than a trace (less than 1 mg) of growth on minimal medium[22] after four days. All are stimulated to grow at the wild type rate by the addition of isoleucine and valine. Uniformly, in the experiments described here, we have used equimolar concentrations of 10 μ moles per 25 ml of L-isoleucine and L-valine.

None of the known precursors of isoleucine and valine that have been tested[23] have supported growth except the keto acid precursors, α-ketoisovaleric acid and α-keto-β-methylvaleric acid. These were effective in combination only for mutants 319, 327, 329, 330, 332, and 305, however.

In general, the *T iv* mutants grow best at an acid pH between 4.5 and 5.8. Some of them grow at pH 7.5, but a number of them do not, as shown in Table 2.

Linkage Relationships.—The mutants were all crossed to one another, in several cases both ways, on supplemented Westergaard–Mitchell Medium.[24] The appropriate and necessary mating types were obtained by crossing the mutants to Em5297a or Em5256A. Wild type prototrophs were determined by the mass plating method of Newmeyer.[25] In general, ascospore germination was about 20 to 50 per cent on the control plates, except for the cross 16117 × 324 in which it was 80 to 90 per cent.

Table 1 gives the results from all crosses which gave perithecium formation and provided sufficient ascospores for testing. The recombination per cent was calculated by dividing the number of observed wild type recombinants by the

TABLE 1

RECOMBINATION VALUES CALCULATED FROM WILD TYPE SEGREGANTS OBSERVED

Cross	Total spores	Per cent recomb.		Cross	Total spores	Per cent recomb.	
318A × 322a	113	0.0	1 × 1	321A × 324a	6,630	0.27	2 × 2
319A × 322a*	972	0.62		327A × 321a*	1,452	0.82	
320A × 322a	500	1.2		324A × 326a	233	1.7	
305A × 324a	1,586	0.0		321A × 330a	1,660	0.96	2 × 3
318A × 321a	632	0.63		321A × 323a*	1,440	3.2	
321A × 325a*	10,260	1.28	1 × 2	16117a × 311A	3,071	0.0	
319A × 321a*	8,732	1.38		16117A × 329a	492	0.0	
318A × 324a*	840	3.34		16117A × 328a	1,300	0.0	
320A × 327a	706	0.0		16117a × 318A	318	0.0	
322A × 323a	606	0.66		16117a × 304A	11,380	0.009	
318A × 323a	1,793	1.0	1 × 3	16117a × 325A	6,720	0.03	
318A × 330a*	2,380	3.4		16117a × 320A	7,080	0.03	
320A × 323a	840	3.8		16117a × 305A	3,540	0.11	
322A × 329a	420	4.3		16117a × 324A	41,619	0.12	
				16117a × 323A	1,188	0.34	

* Segregants not checked for pseudo-wild types.

number of total germinated ascospores on the control plates and multiplying by 200. The apparent wild type segregants were isolated and, in most cases, conidia or hyphal tips were tested to determine whether they were pseudo-wild types.[26] Those segregants classified as pseudo-wild types were subtracted before determining the recombination per cent. The results are listed in Table 1 with the protoperithecial parent listed first.

It is evident from the data in Table 1 that all of the *T iv* mutants which showed fertility in crosses are closely linked to 16117 and to one another. The recombination values obtained range from 0 to 4 per cent. Those values obtained from crosses to 16117 were uniformly lower than from other crosses. The high recombination values received in some of the *T iv* × *T iv* crosses may be a result of poor germination of the mutant segregant ascospores.

Mutant 16117 is located in linkage group V in *Neurospora crassa*, between *spray* (B132) and *lysine-1* (33933).[27] We have verified this for our stock of 16117 with markers obtained from Dr. David Perkins of Stanford University.

Two mutants, 331 and 332 did not cross to any of the others including 16117. These were crossed to both *spray* and *lysine-1*, and found to be located in the same approximate position as 16117 between these two markers.

Complementation in Heterocaryons.—Since the isoleucine-valine mutants described here are both closely linked or "allelic" and are all obviously closely related biochemically, they provide ideal material for complementation studies aimed at the analysis of structure.

Heterocaryons were formed between the nineteen mutants by various procedures, but the method which gave the most consistent results was that described by de Serres.[28] Heavy conidial suspensions (but not mycelial clumps) from each of two mutant strains to be tested were pipetted in small spots at the periphery of a minimal agar surface in a petri dish. Both control and mixture spots were made. Heterocaryon formation and complementation is indicated by growth from the mixture spot, but not from the control spots. Incubation was carried out for ten days at a temperature of 25°C. Certain of the isoleucine-valine mutants described here are leaky, i.e., show small growth on minimal. Indication of complementation on minimal, in tests involving one or both participants with leaky propensities, was considered positive only when the heterocaryon started definite growth before the controls.

The data obtained from the complementation tests were found to fit a complementation matrix which puts them in dictionary order. The application of this technique, described by Benzer for recombination data from phage,[5] enables one to determine whether the elements one is dealing with can be arranged in a linear array, and, if so, in what order.

Figure 1 gives the results from the complementation tests. It shows which mutants complement and which do not following the usual convention[16] of showing overlapping lines or bars when complementation does not occur. Furthermore, Figure 1 shows that 325, 322, 320, 319, 305, 318, 304, 327, 321, 324, 330, 328, 329, 323, 311, 332, and 16117 are dictated to that order by the complementation data. The data are not complete enough for the precise placement of 331 and 326, but they can be assumed to occupy any position between that below 319 and above 327 and 321. They are placed between 304 and 327 for reasons discussed below.

The order may also be modified somewhat for certain of the others when more detailed data are obtained. However, the data are sufficient to demonstrate an order, and to demonstrate that there is one group, consisting of 325 through 326 in Figure 1, within which there is no complementation. This group can be placed to the left of the group 327 through 16117. In the latter group there is complementation between certain members of the group as well as between them and members of the first group.

Although we have not yet made detailed determinations of the growth rates of the heterocaryons on minimal, and the time of their appearance from a mixture

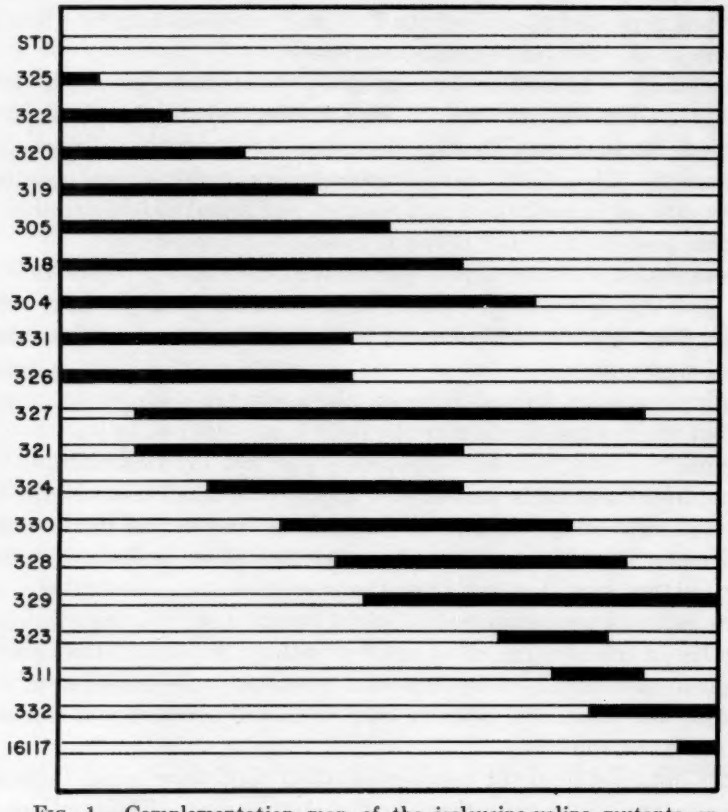

FIG. 1.—Complementation map of the isoleucine-valine mutants on the Vth Chromosome of *Neurospora crassa* which are closely linked to 16117.

of conidia, it is clear that differences in growth rate and time of appearance do occur. For example, those at the top of the list, 325 through 318, form complementing heterocaryons with certain of those toward the bottom, i.e., 323, 311, 16117, and 324, which grow almost as fast as wild type.

It can be concluded from the complementation data that there are at least 18 discernibly different mutants at this locus, and that these mutants can be placed in a linear array which indicates the areas in which they show blemishes or lesions. (See Benzer.[5])

Biochemical and Physiological Properties of the Mutants.—The properties of

two of the mutants, 16117 and 304, have been partially described before.[29-31] Briefly, as a result of the previous observations, it has been postulated that 16117 is blocked at the conversion of the dihydroxy acids, α,β-dihydroxyisovaleric acid and α,β-dihydroxy-β-methylvaleric acid, to the corresponding α-keto acids, and that 304 is blocked at the conversion of the β-keto acids, α-acetolactic acid, and α-aceto-α-hydroxybutyric acid, to the dihydroxy acids by the absence or partial inactivation of an enzyme, reductoisomerase.[32]

All of the mutants here have been investigated to determine whether they accumulate carbinols[33] and pyruvate,[34] a characteristic of the type of block found in 304, or show the dihydroxy acid accumulation characteristic of 16117. In addition, the dihydroxy acid dehydrase activities[35] of crude extracts derived from them were determined.

Table 2 presents the results in qualitative form. A minus sign in the carbinol column indicates that the carbinol accumulation noted after four days' growth is equivalent to, or at most about 10 times above, the wild type level. Where a plus is indicated it means that there is at least 100 to 2,000 times more carbinol accumulated by that strain under the same conditions. The identity of the carbinols accumulated by these strains was determined by the formation and identi-

TABLE 2

BIOCHEMICAL AND PHYSIOLOGICAL DIFFERENCES IN THE MUTANT STRAINS

	Strain	Accumulations			Dehydrase activity	Growth at pH 7.5
		Carbinols	Pyruvate	Dihydroxy acids		
1	325	+	+	−	high	−
	322	+	+	−	high	−
	320	+	+	−	high	−
	319	+	+	−	high	−
	305	+	+	−	high	−
	318	+	+	−	high	+
2	304	+	+	+	high	−
	331	+	+	+	high	−
	326	−	−	+	low	+
	327	−	−	+	low	+
	321	+	+	+	high	−
	324	+	+	+	high	−
3	330	−	−	+	low	+
	328	−	−	+	low	+
	329	−	−	+	low	+
	323	−	−	+	low	+
	311	−	−	+	low	+
	332	−	−	+	low	+
	16117	−	+	+	low	+

fication of the pteridines.[30] In all instances the high carbinol mutants were found to accumulate both acetylmethylcarbinol and acetylethylcarbinol. Pyruvate accumulation by those strains marked plus in the pyruvate column is at least 5 to 100 times higher than those marked minus. The minus strains all accumulate pyruvate at the wild type level. The presence or absence of dihydroxy acids was determined qualitatively after ether extraction of the acids from the concentrated, acidified medium, and chromatographing the ether soluble material on paper. The dihydroxy acid spots were developed with periodate.[31] The ether extractions were quantitative and from equal amounts of media from the various mutants. The extracts were spotted quantitatively on the paper before chromatography. Quantitative estimates were made of the amounts of dihydroxy acids

accumulated by 304 and those listed below it in Table 2 were made and will be reported elsewhere. Of these, 304 and 324 accumulated α,β-dihydroxyisovaleric acid only. The rest of the mutants accumulated both acids. Mutants 325 through 318 did not accumulate dihydroxy acids detectable by this method.

The dehydrase specific activity was determined by the method described by Myers and Adelberg.[35] Those mutants indicated as having high dehydrase activity were as high or higher than wild type. The low dehydrase containing mutants had 10 to 50 per cent of the activity found in the high dehydrase strains. There was no overlap in the dehydrase activity values between the high and low strains.

All mutants were found to have a hydroxy keto acid reductase activity as high or higher than wild type.[31]

A further difference between the mutants was found on determining their growth in isoleucine and valine supplemented medium at different conditions of pH. None of the mutants indicated with a minus sign in Table 2 grows at a pH of 7.5. Those mutants which grow at pH 7.5 grow equally well at pH 5.8 or 4.5.

Table 2 shows that a sharp distinction can be made between mutants using the criteria indicated. Strains 325 through 318 form a group with similar properties but are distinctly different from 326, 327, and 320 through 16117. Four mutants 304, 331, 321, and 324 have some characteristics in common with both groups, and hence are somewhat intermediate.

Correlation of Biochemical, Complementation, and Linkage Data.—The mutants have been arranged in the same order in Table 2 as they occupy in the complementation map in Figure 1. It is obvious that there is considerable correlation. The high carbinol, pyruvate, dehydrase mutants tend to the left end of the map and the high dihydroxy acid, low dehydrase mutants to the right end. There is also a correlation with pH effect.

For purposes of discussion the mutants have been placed in three groups (1, 2, and 3) as indicated in Table 2. Crosses of Group 1 $\times$ Group 1 mutants designated as 1 $\times$ 1 in Table 1, show some indication of closer linkage between mutants within the group than with some of the mutants outside the group. Compare 1 $\times$ 1 crosses to 1 $\times$ 2 and 1 $\times$ 3 crosses, for example. The data are obviously not sufficient to make any definite conclusion about a correlation between the linkage map, the complementation map, and the biochemical characteristics of the mutants. They are, however, sufficiently comprehensive to prove close linkage, and to indicate the possibility of a linear arrangement.

Discussion.—From the relatively limited surveys of the biochemical characteristics of the isoleucine-valine mutants described here made up to the present time, it would appear that the group 1 mutants are blocked at the reaction by which the β-keto acids are converted to the dihydroxy acids, and the group 3 mutants are blocked at the step at which the dihydroxy acids are converted to the α-keto acids. (See Figure 2.) The present evidence for this is that the group 1 mutants accumulate large quantities of the carbinols, acetylmethylcarbinol (AMC) and acetylethylcarbinol (AEC), and the group 3 mutants together with 326 and 327 accumulate dihydroxy acids and have low dehydrase activity. The accumulation of the carbinols, AMC and AEC, would be expected if the reductoisomerase indicated in Figure 2 were inoperative or low in activity, since AMC and AEC

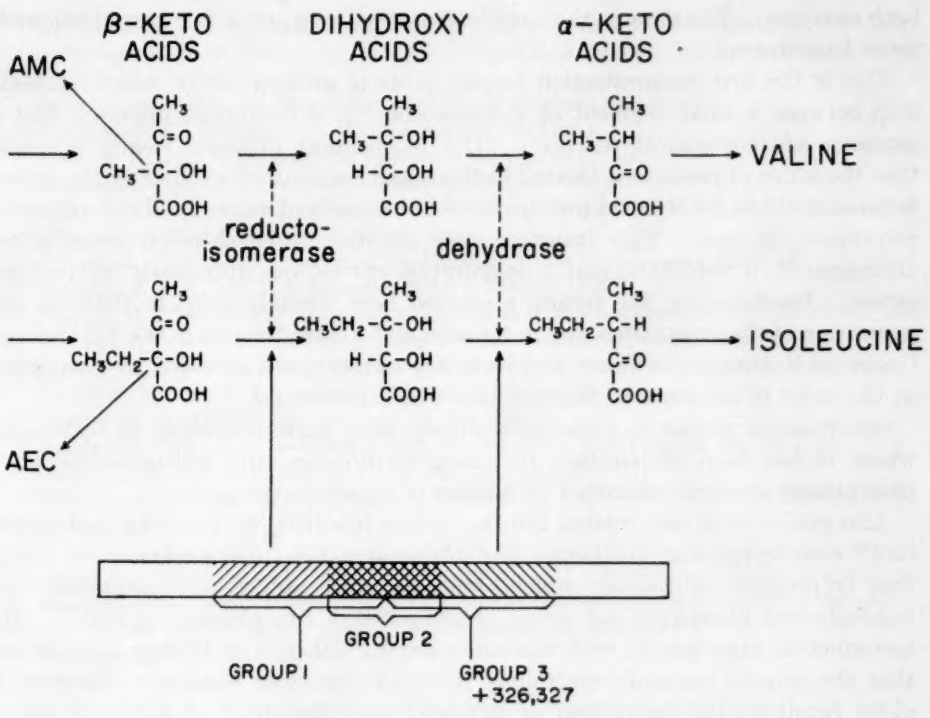

FIG. 2.—Hypothesized relationship between genetic and biochemical pathway.

are the metabolic end products expected from accumulating β-keto acids. At least one of the mutants in group 3 has been shown to possess an active reductoisomerase in crude extracts. The others have not been tested, but presumably they have it, since they synthesize the dihydroxy acids *in vivo*.

The mutants in group 2, 304, 331, 321, 324, present a puzzle since they accumulate significant quantities of both carbinols and dihydroxy acids. Possibly they are deficient in both enzymes, but the reductoisomerase is sufficiently active to allow enough leakage during the course of growth to form detectable dihydroxy acids. One mutant, 304, has been tested for the overall enzyme and found not to have detectable activity. The results, reported elsewhere,[31] are not, however, to be considered conclusive, since conditions for the proper demonstration of the reductoisomerase in crude extracts of *Neurospora* have yet to be worked out. Purified preparations of this enzyme from *Neurospora* have been made, however.

In Figure 2 we have indicated what we believe may be the relationship between the mutations and the sequence of reactions. The hatched area represents the area of the chromosome affected by the mutations. We hypothesize that the group 1 and 3 mutations and 326 and 327 affect the area indicated by the brackets, and hence alter only one or the other of the two enzymes. The group 2 mutants, 304, 331, 321, and 324, because of their biochemical properties and intermediate position in the complementation map, we tentatively place in the middle. We suggest that these mutations overlap with the two outside areas and hence affect

both enzymes. The data in the complementation map are in fair agreement with these hypotheses.

This is the first demonstration known to us of an apparently direct relationship between a small segment of a chromosome in a eucaryotic organism and a sequence of biochemical reactions. The biochemical evidence clearly suggests that the series of mutations located in this small segment affect directly the transformation of the β-keto acid precursors of isoleucine and valine to the α-keto acid precursors, at least. This transformation involves three chemical steps, a rearrangement, a reduction, and a dehydration carried out apparently by two enzymes. Furthermore, the results presented here strongly indicate that the organization of the genetic material in the area under consideration in the Vth Linkage Group in *Neurospora* is linear, and its active elements are arranged to correspond to the order of sequence of the reactions which it controls.

Observations similar to these have already been made previously in *Salmonella* where it has been shown that histidine, tryptophan, and isoleucine-valine[8-11] biosynthesis are each controlled by a series of closely linked genes.

Also probably closely related are the results reported by Yanofsky and Crawford[36] with tryptophan synthetase in *Escherichia coli*. These workers have found that tryptophan synthetase, which catalyzes the synthesis of tryptophan from indoleglycerol phosphate and serine, is composed of two elements A and B. By transduction experiments with mutants carrying either A or B they have shown that the genetic elements controlling A and B are close together. Fraction A alone catalyzes the conversion of indoleglycerol phosphate to indole + triose phosphate while B alone catalyzes the formation of tryptophan from indole and serine. When A and B are together, they have maximal activity for carrying out the synthesis of tryptophan from indoleglycerol phosphate and serine without the evident appearance of free indole. The similarity to the isoleucine-valine system described here exists in that the formation of the dihydroxy acids from the β-keto acids by (1) a rearrangement to form the α-keto-β-hydroxy acids and (2) the reduction of these to form the α,β-dihydroxy acids, and the accumulation of the α-keto-β-hydroxy acids cannot be detected when the reductoisomerase is catalyzing the reaction. However, purified reductoisomerase with no initial reductase activity for the α-keto-β-hydroxy acids as initial substrates, gains such activity for reduction alone after standing and concommitantly loses overall reductoisomerase activity.[37]

Summary.—A group of mutants of *Neurospora crassa* requiring isoleucine plus valine is described. The mutants are all shown to be in linkage group V and located in a segment probably not more than four map units in length. The results of complementation experiments are described, and it is shown that a correlation can be made between the biochemical properties of the mutants and their indicated positions in the complementation map.

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† Predoctoral Fellow of the National Institutes of Health.

[1] Oliver, C. P., these PROCEEDINGS, **26**, 452 (1940).

[2] Green, M. M., and K. C. Green, these PROCEEDINGS, **35**, 586 (1949).

[3] Lewis, E. B., *Genetics*, **30**, 137 (1945).

[4] Pontecorvo, G., in *Trends in Genetic Analysis* (New York: Columbia University Press, 1958).

[5] Benzer, S., these PROCEEDINGS, **45**, 1607 (1959).

[6] Hotchkiss, R. D., and A. H. Evans, *Cold Spring Harbor Symp. Quant. Biol.*, **23**, 85 (1958).

[7] Lederberg, J., E. M. Lederberg, N. D. Zinder, and E. R. Lively, *Cold Spring Harbor Symp. Quant. Biol.*, **16**, 413 (1951).

[8] Demerec, M., and Z. Hartman, Carnegie Inst. Wash. Publ. **612**, 5 (1956).

[9] Hartman, P. E., Carnegie Inst. Wash. Publ. **612**, 35 (1956).

[10] Ames, B. N., and B. Garry, these PROCEEDINGS, **45**, 1453 (1959).

[11] Wagner, R. P., and A. Bergquist (in manuscript).

[12] Benzer, A., in *A Symposium on the Chemical Basis of Heredity*, ed. by W. D. McElroy and B. Glass (Baltimore: The Johns Hopkins Press, 1957).

[13] Catcheside, D. G., and A. Overton, *Cold Spring Harbor Symp. Quant. Biol.*, **23**, 137 (1958).

[14] Fincham, J. R. S., and J. A. Pateman, *Nature*, **179**, 741 (1957).

[15] Giles, N. H., C. W. H. Partridge, and N. J. Nelson, these PROCEEDINGS, **43**, 305 (1957).

[16] Woodward, D. O., C. W. H. Partridge, and N. H. Giles, these PROCEEDINGS, **44**, 1237 (1958).

[17] Case, M. E. and N. H. Giles, *Cold Spring Harbor Symp. Quantitative Biol.*, **23**, 119 (1958).

[18] Beadle, G. W., and V. L. Coonradt, *Genetics*, **29**, 291 (1944).

[19] Woodward, D. O., these PROCEEDINGS, **45**, 846 (1959).

[20] Woodward, V. W., J. R. de Zeeuw, and A. M. Srb, these PROCEEDINGS, **40**, 192 (1954).

[21] Beadle, G. W., and E. L. Tatum, *Am. J. Bot.*, **32**, 678 (1945).

[22] The minimal medium used here was that described by Vogel, H. J., *Microbial Genetics Bull.*, **13**, 42.

[23] α-aceto-α-hydroxybutyric acid, α-acetolactic acid, α-keto-β-hydroxy-β-methylvaleric acid, α-keto-β-hydroxyisovaleric acid, α,β-dihydroxyisovaleric acid, α,β-dihydroxy-β-methylvaleric acid and the α-keto acids were tested by an auxonographic method to be described in detail elsewhere.

[24] Westergaard, M., and H. K. Mitchell, *Am. J. Botany*, **34**, 573 (1947).

[25] Newmeyer, D., *Genetics*, **39**, 604 (1954).

[26] Pittinger, T., *Genetics*, **39**, 326 (1954).

[27] Strickland, W. N., D. D. Perkins, and C. C. Veatch, *Genetics*, **44**, 1221 (1959).

[28] de Serres, F. J., *Genetics*, **41**, 668 (1956).

[29] Adelberg, E. A., D. Bonner, and E. L. Tatum, *J. Biol. Chem.*, **190**, 837 (1951).

[30] Wagner, R. P., A. Bergquist, and H. S. Forrest, *J. Biol. Chem.*, **234**, 99 (1959).

[31] Wagner, R. P., A. N. Radhakrishnan, and E. E. Snell, these PROCEEDINGS, **44**, 1047 (1958).

[32] Radhakrishnan, A. N., R. P. Wagner, and E. E. Snell (in manuscript).

[33] Total carbinols determined by the method of Westerfeld, W. W., *J. Biol. Chem.*, **161**, 495 (1945).

[34] Pyruvate was identified qualitatively by chromatography of the 2,4-dinitrophenylhydrozone and quantitatively by the method of Friedemann, T. E. and J. E. Haugen, *J. Biol. Chem.*, **147**, 415 (1943).

[35] Myers, J. W., and E. A. Adelberg, these PROCEEDINGS, **40**, 493 (1954).

[36] Yanofsky, C., and I. P. Crawford, these PROCEEDINGS, **45**, 1016 (1959).

[37] Armstrong, F. B., and R. P. Wagner, unpublished results.

SOME MATHEMATICAL ASPECTS OF OPTIMAL PREDATION IN ECOLOGY AND BOVICULTURE

By Richard Bellman and Robert Kalaba

THE RAND CORPORATION, SANTA MONICA, CALIFORNIA

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General mathematical problems arising in the scientific study of predation have been studied from a variety of viewpoints. Primary emphasis has been given to the descriptive aspects of the prey and predator populations under various assumptions concerning interactions among the different members of the populations during these processes, and to birth and death processes. In particular, we wish to call attention to the recent paper by MacArthur,[1] where additional references can be found, as well as to the classical works of Volterra,[2] Lotka,[3] and Chiang.[6]

The major objective of this note is to show how the functional equation technique of a new mathematical discipline, dynamic programming[4], can be used in formulating and solving—both analytically and numerically—a variety of problems of optimal predation. We wish to determine optimal predation policies and are thus interested in the *control*, as opposed to the *descriptive*, aspects of predation processes.

An Ecological Process.—Let us suppose that members of two different populations, called type I and type II, are present, and that members of type I prey on members of type II, but not conversely. Let us furthermore suppose that the presence of neither type is desirable (e.g., bacterial populations), and that we possess a drug (or other technique such as radiation) which is effective in destroying members of population type I, but is ineffective against those of type II. Our task is to determine the most efficacious mode of administration of the drug in an effort to control the two populations. The problem is not trivial since the administration of maximal dosages of the "drug" may deplete the type I population to such an extent that type II population may become dangerously large.

To cast such a problem in more mathematical form let us introduce a bit of nomenclature:

$$u(t) = \text{size of population I at time } t, \tag{1}$$

$$v(t) = \text{size of population II at time } t, \tag{2}$$

$$w(t) = \text{rate of administration of the drug at time } t. \tag{3}$$

We then consider the populations to be described by the differential equations and initial conditions

$$\begin{aligned} \dot{u} &= k_1(w(t))u, \; u(0) = c_1, \\ \dot{v} &= k_2(w(t))v - k_3(w(t))u, \; v(0) = c_2, \end{aligned} \tag{4}$$

which hold for $u, v \geq 0$ and for $0 \leq t \leq T$, where T is the duration of the process. We measure the undesirability of the two populations at any time t by the expression $a_1u(t) + a_2v(t)$. Our objective is to determine $w = w(t)$, $0 \leq t \leq T$, in such a manner that we make the functional

$$\max_{0 \leq t \leq T} \{a_1u(t) + a_2v(t)\} \tag{5}$$

be as small as possible. In place of the usual forcing term effect of control, we assume in writing equations (4) that the influence of the drug is to affect the growth and interactions of the two populations.

We introduce the function $f(c_1, c_2, T)$, defined by the relationship

$$f(c_1, c_2, T) = \min_{w} \max_{0 \le t < T} \{a_1 u(t) + a_2 v(t)\}, \tag{6}$$

and use the principle of optimality[4] to derive the functional equation

$$f(c_1, c_2, T) = \max [a_1 c_1 + a_2 c_2, \min_{w} f(c_1 + k_1(w)c_1 h, c_2 + k_2(w)c_2 h - k_3(w)c_1 h, T - h)] + 0(h). \tag{7}$$

This can be used for computational purposes, in conjunction with the terminal condition

$$f(c_1, c_2, 0) = a_1 c_1 + a_2 c_2, \tag{8}$$

or can be used as the basis for further analytical studies. Lastly, we remark that the function $f(c_1, c_2, T)$ is homogeneous of degree one in c_1 and c_2, a fact which can be used to advantage computationally to reduce the dimension of f from two to one.

Boviculture.—Suppose we have a herd of cattle with x_i head of age i, $i = 0, 1, 2, \ldots, K - 1$, and x_K head of age K or older. Each year we can either send some of the cattle to market, each individual of age i being of worth a_i, or we can keep them to build up the herd. We suppose that x_i members of age i give rise to $b_i x_i$ calves in a year and that a_i is the fraction of individuals of age i surviving to age $i + 1$. For an N-stage process, $N = 1, 2, \ldots$, we wish to find the optimal breeding policy, that is, the policy that results in maximal over-all return. We shall assume that the last decision must be to send all cattle to market.

We introduce the sequence of functions

(1) $f_N(x_0, x_1, x_2, \ldots, x_K)$ = the return from an N-stage process beginning with x_i cattle of age i and using an optimal predation policy, $N = 1, 2, \ldots$.

First we observe that

$$(2) \quad f_1(x_0, x_1, x_2, \ldots, x_K) = \sum_{i=0}^{K} a_i x_i,$$

and then we use the principle of optimality to derive the functional relationships

$$(3) \quad f_{k+1}(x_0, x_1, \ldots, x_K) = \max_{\substack{0 \le y_j \le x_j \\ j = 0, 1, 2, \ldots, K}} \left\{ \sum_{i=0}^{K} a_j y_j + f_k \left(\sum_{j=0}^{K} b_j(x_j - y_j), a_0(x_0 - y_0), \ldots, a_{N-2}x_{N-2}, a_{N-1}x_{N-1} + a_N x_N \right) \right\},$$

valid for $k = 1, 2, \ldots$.

The sequence of functions $f_1, f_2, \ldots$ is determined recursively, beginning with f_1. Simultaneously, we determine the optimal values of y_i, which tell us how many cows in each age group to send to market in terms of the current composition of the herd and the time remaining in the process.

Problems of this nature can be solved analytically in a number of cases (cf. Bellman, Glicksberg, Gross[7]).

Discussion.—The two simplified models which have been sketched here serve but to suggest what can be done with regard to predation and breeding processes using modern mathematical techniques and employing high-speed digital machines. Additional papers, in which a variety of stochastic and adaptive[5] features are incorporated, will be prepared.

We wish to acknowledge the fruitful discussions that we have had with I. Cooper, J. Digby, J. Jacquez, and J. Lyman.

[1] MacArthur, R., "On the relation between reproductive value and optimal predation," these PROCEEDINGS, **46,** 143–145 (1960).

[2] Volterra, V., *Theory of Functionals and of Integral and Integrodifferential Equations* (New York: Dover Publ. Inc., 1959).

[3] Lotka, A., *Elements of Mathematical Biology,* (New York: Dover Publ. Inc., 1957).

[4] Bellman, R., *Dynamic Programming* (Princeton: Princeton Univ. Press, 1957).

[5] Bellman, R., and R. Kalaba, "A mathematical theory of adaptive control processes," these PROCEEDINGS, **45,** 1288–1290 (1959).

[6] Chiang, C., "Competition and other interactions between species," in *Statistics and Mathematics in Biology,* ed. Kempthorne *et al.*(Ames: Iowa State College Press, 1954).

[7] Bellman, R., I. Glicksberg, and O. Gross, *Some Aspects of the Mathematical Theory of Control Processes,* The RAND Corporation, Report R-313 (1958).

CLASSES OF HOLOMORPHIC FUNCTIONS OF SEVERAL VARIABLES IN CIRCULAR DOMAINS*

By S. Bochner

PRINCETON UNIVERSITY

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We will extend a theorem of (F. and M. Riesz and) Hardy-Littlewood[1] from holomorphic functions $f(z)$ in the unit disk $|z| < 1$ to holomorphic functions of several variables $f(z_1, \ldots, z_k)$ in general circular domains.

THEOREM 1.[1] *If $f(z)$ is holomorphic in $|z| < 1$ and, for some $\lambda > 0$,*

$$\sup_{0<r<1} \int_0^1 |f(re^{2\pi i\theta})|^\lambda \, d\theta \equiv C^\lambda < \infty,$$

then

$$\int_0^1 \sup_{0<r<1} |f(re^{2\pi i\theta})|^\lambda \, d\theta \leq \alpha_\lambda C^\lambda,$$

where α_λ depends only on λ and nothing else.

Also, for $r_1, r_2 < 1$,

$$\int_0^1 |f(r_1e^{2\pi i\vartheta}) - f(r_2e^{2\pi i\theta})|^\lambda \, d\theta \to 0 \text{ as } (r_1, r_2) \to (1, 1),$$

and there exists a measurable function $F(\theta)$ on the boundary $\zeta = e^{2\pi i\theta}$ such that, for $0 < r < 1$,

$$\int_0^1 |f(re^{2\pi i\theta}) - F(\theta)|^\lambda \, d\theta \to 0 \text{ as } r \to 1.$$

The quantity

$$M(r) = \int_0^1 |f(re^{2\pi i\theta})|^\lambda \, d\theta$$

is monotonely increasing in r. Using this, it is not hard to verify that Theorem 1 gives rise to the following generalization of itself.

THEOREM 2. *If our holomorphic function depends on a parameter ξ, $f(z) = \varphi(z; \xi)$; if ξ is the point of a Lebesgue measure space $\{X: (\xi), d\mu(\xi)\}$ and $\varphi(z; \xi)$ is measurable in $(z; \xi)$; if for almost all ξ, $\varphi(z; \xi)$ is holomorphic in $|z| < 1$; and if, for $\lambda > 0$,*

$$\sup_{0<r<1} \int_X d\xi \int_0^1 |\varphi(re^{2\pi i\theta}; \xi)|^\lambda \, d\theta \equiv C^\lambda < \infty,$$

then, for the same α_λ,

$$\int_X d\xi \int_0^1 \sup_{0<r<1} |\varphi(re^{2\pi i\theta}; \xi)|^\lambda \, d\theta \leq \alpha_\lambda C^\lambda;$$

also,

$$\int_X d\xi \int_0^1 |\varphi(r_1e^{2\pi i\theta}; \xi) - \varphi(r_2e^{2\pi i\theta}; \xi)|^\lambda \, d\theta \to 0 \text{ as } (r_1, r_2) \to (1, 1)$$

and there exists a function $\Phi(\theta; \xi)$ measurable in $(\theta; \xi)$ such that

$$x \, d\xi \int_0^1 |\varphi(re^{2\pi i\theta}; \xi) - \Phi(\theta; \xi)|^\lambda \, d\theta \to 0.$$

Now, in the space of k complex variables $(z_1, \ldots, z_k)$ let D be a (bounded) circular domain containing the origin. Thus, if $(a_1, \ldots, a_k) \epsilon D$, then also $(za_1, \ldots, za_k) \epsilon D$ for $|z| \leq 1$. Let B denote the boundary of B with relative topology. We take on B any finite Borel measure $\omega(B_0)$, $B_0 \subset B$, subject only to the condition that it be circularly invariant. By this we mean that if we replace all points $(\zeta_1, \ldots, \zeta_k)$ of B_0 by $(e^{2\pi i\theta} \zeta_1, \ldots, e^{2\pi i\theta} \zeta_k)$ and denote the new set by B_0^θ then $\omega(B_0^\theta) = \omega(B_0)$. The leading feature of such a measure is the following. If $\Psi(\zeta_1, \ldots, \zeta_k)$ is a (non-holomorphic) function on B summable with respect to $\omega(B_0)$ then

$$\int_B \Psi(\zeta_1, \ldots, \zeta_k)d\omega = \int_0^1 d\theta \int_B \Psi(e^{2\pi i\theta} \zeta_1, \ldots, e^{2\pi i\theta} \zeta_k)d\omega.$$

Because of this, Theorem 2 implies as follows.

THEOREM 3. *If $f(z_1, \ldots, z_k)$ is holomorphic in a bounded circular domain D with origin, and if*

$$\sup_{0 < r < 1} \int_B |f(r\zeta_1, \ldots, r\zeta_k)|^\lambda \, d\omega(\zeta) \equiv C^\lambda < \infty,$$

$\lambda > 0$, relative to a finite circularly invariant Borel measure $\omega(B_0)$ on the boundary B of D; then

$$\int_B \sup_{0 < r < 1} |f(r\zeta_1, \ldots, r\zeta_k)|^\lambda \, d\omega(\zeta) \leq a_\lambda C^\lambda.$$

Also

$$\int_B |f(r_1\zeta_j) - f(r_2\zeta_j)|^\lambda \, d\omega(\zeta) \to 0 \text{ as } (r_1, r_2) \to (1, 1)$$

and there exists a function $F(\zeta_j) \equiv F(\zeta_1, \ldots, \zeta_k)$ on B, measurable relative to $\omega(B_0)$ such that

$$\int_B |f(r\zeta_j) - F(\zeta_j)|^\lambda d\omega(\zeta) \to 0 \text{ as } r \to 1.$$

Theorem 3 applies in particular[2] to the sphere

$$S_k: \quad |z_1|^2 + \ldots + |z_k|^2 < 1$$

with "ordinary" $(2k\text{-}1)$-dimensional surface area $\omega(B_0)$; and it also applies to the polycylinder

$$P_k: \quad |z_1| < 1, \ldots, |z_k| < 1,$$

if the measure $\omega(B_0)$ on its boundary B is defined in such a manner that

$$\int_B \Psi(\zeta_1, \ldots, \zeta_k)d\omega(\zeta) = \int_0^1 \ldots \int_0^1 \Psi(e^{2\pi i\theta_1}, \ldots, e^{2\pi i\theta_k})d\theta_1 \ldots d\theta_k.$$

This measure is concentrated entirely on the k-dimensional subset $|\zeta_1| = 1, \ldots, |\zeta_k| = 1$ of the total $(2k\text{-}1)$-dimensional boundary, but it is a finite Borel measure and circularly invariant, and this was all we have required of it.

We note the corollary ($\lambda = 1$) that if $f(z_1, \ldots, z_k)$ is holomorphic in P_k and

$$\sup_{0 < r < 1} \int_0^1 \ldots \int_0^1 |f(re^{2\pi i\theta_1}, \ldots, re^{2\pi i\theta_k})| \, d\theta_1 \ldots d\theta_k < \infty$$

then there exists a multiperiodic function $F(\theta_j) \equiv F(\theta_1, \ldots, \theta_k)$ of class L_1 such that

$$\int_0^1 \ldots \int_0^1 |f(r\theta_j) - F(\theta_j)| \, d\theta_1 \ldots d\theta_k \to 0, \; r \to 1.$$

This non-trivial proposition was originally stated and proved by this author,[3] and the proof employed the radial maximum of Hardy-Littlewood, but in a much more complicated manner than the present approach suggests. Also an entirely different proof of this "corollary" was recently given by Helson and Lowdenslager,[4] and we wish to point out that our original proof and their recent method both yield a somewhat stronger result which cannot be easily stated within the present set-up.

Finally we observe that for $\lambda > 1$ we have the following theorem again obtainable from its one variable prototype.[1]

THEOREM 4. *For $\lambda > 1$ all parts of Theorem 3 (and also of Theorem 2) are valid if instead of a holomorphic function in D we consider a function $u(z_1, \ldots, z_k)$ which is the real part of a holomorphic function.*

Also, for $\lambda = 1$ there remains the statement that

$$\sup_{0 < r < 1} \int_B |u(r\zeta_j)| \log^+ |u(r\zeta_j)| d\omega(\zeta) \leq C < \infty$$

implies the relation

$$\int_B \sup_{0 < r < 1} |u(r\zeta_j)| d\omega(\zeta) \leq \alpha C + \beta$$

and the existence of a function $U(\zeta_j)$ for which

$$\int_B |u(r\zeta_j) - U(\zeta_j)| d\omega(\zeta) \to 0, \; r \to 1.$$

Remark: Note that the log factor in the assumption appears in first power for all complex dimensions k.

One can also extend a theorem of M. Riesz[5] on conjugate functions and the statement is as follows.

THEOREM 5. *If $f(z_j) = u(z_j) + iv(z_j)$ is holomorphic in D and $v(0) = 0$, then, for $p > 1$,*

$$\sup_r \int_B |v(r\zeta_j)|^p d\omega \leq \gamma_p \sup_r \int_B |u(r\zeta_j)|^p d\omega$$

and, for $p = 1$,

$$\sup_r \int_B |v(r\zeta_j)| d\omega \leq \gamma \sup_r \int_B |u(r\zeta_j)| \log^+ |u| d\omega + \delta,$$

where γ_p, γ, δ are fixed constants.

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[1] Hardy, G. H., and J. E. Littlewood, "A maximal theorem with function-theoretic applications," *Acta Mathematica*, **54**, 81–116 (1930).

[2] Rauch, H. E., "Harmonic and analytic functions of several variables and the maximal theorem of Hardy and Littlewood," *Canadian Journal of Mathematics*, **8**, 171–183 (1956); see also Smith, K. T., "A generalization of an inequality of Hardy and Littlewood," *ibid.*, 157–170 (1956).

[3] Bochner, S., "Boundary values of analytic functions in several variables and of almost periodic functions," *Annals of Mathematics*, **45**, 708–722 (1944).

[4] Helson, H., and D. Lowdenslager, "Prediction theory and Fourier Series in several variables," *Acta Mathematica*, **99**, 165–202 (1958).

[5] Riesz, M., "Sur les fonctions conjugueés," *Mathematische Zeitschrift*, **27**, 218–244 (1928).

BETTI AND PICARD NUMBERS OF ABSTRACT ALGEBRAIC SURFACES

By Jun-ichi Igusa*

INSTITUTE FOR ADVANCED STUDY AND JOHNS HOPKINS UNIVERSITY

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1. *Introduction.*—If V is a non-singular (irreducible) algebraic surface, we can define its "good Betti numbers" in the following way. We take both B_0 and B_4 to be equal to 1. Also, we take B_1 and B_3 to be the twice of the dimension of the Picard variety of V. Finally, we define B_2 so that we get

$$\Sigma_i(-1)^i B_i = \chi \ (= \text{Euler number of } V).$$

The definition is not vacuous, because χ has an independent meaning. In fact $\chi - 4$ is known as Zeuthen–Segre's invariant of V.[1] We observe, however, that this definition leaves uncertain whether B_2 is positive or not. In this short note, we shall outline a proof of the *theorem* stating that our *two-dimensional Betti number B_2 is at least equal to the Picard number ρ of V.* This fact can be looked upon as giving a precise upper bound to the Picard number. It might be worthwhile to investigate the possibility of applying our method to absolute surfaces in connection with the rank problem of elliptic curves or, more generally, of Abelian varieties.

2. *Preliminary Observations.*—Let ω be a linear differential on V different from 0. Then, we can associate an 0-cycle

$$<\omega> = \Sigma_{P \in V} m_P(\omega) \cdot P$$

to ω in the following way. We take local parameters t_1, t_2 of V at P and we write ω in the form $f \cdot (h_1 dt_1 + h_2 dt_2)$ by three functions f, h_1, h_2 on V such that h_1, h_2 are holomorphic and have no zero curve in common at P. Then, the multiplicity of P in the intersection of divisors (h_1), (h_2) of h_1, h_2 depends only on P and ω. This we take as $m_P(\omega)$. On the other hand, let Ω be a double differential on V different from 0. We shall use the standard notation (ω), (Ω) to denote divisors of the differentials ω, Ω. Then, we can consider $<\omega> + (\omega) \cdot (\Omega) - (\omega) \cdot (\omega)$ as defining a rational equivalence class on V.[2] The point is that this class is intrinsic, i.e., it does not depend on the choice of ω and Ω. The proof is left to the reader. Actually, we can show that this class is the second canonical class of V. At any rate, if we take its degree, i.e., if we pass to algebraic equivalence, we get a relative invariant χ. There is a classical way of calculating this number.

We have shown elsewhere[3] that we can find a function f on V with values in a numerical straight-line D such that (*i*) every fiber $C_u = f^{-1}(u)$ is absolutely irreducible; (*ii*) there exists a finite number of points, say $a_1, \ldots, a_\alpha$, on D such that the fibers become singular only at these points; (*iii*) any two fibers are transversal to each other at a finite number of points, say, $b_1, \ldots, b_\beta$. We note that, if $d_1, \ldots, d_\alpha$ are the double points of fibers over $a_1, \ldots, a_\alpha$, we have $<df> = \Sigma_i d_i + \Sigma_j b_j$. Moreover, if we denote the constant arithmetic genus of C_u by g, the degree of the rational equivalence class $((\Omega) + (f)_\infty) \cdot (f)_\infty$ is $2g - 2$. Since we also have $(df) = -2(f)_\infty$, we get

$$\chi = \alpha - \beta - 4g + 4.$$

We need another observation. Let k be an arbitrary algebraically closed field of definition of V and f. Let u be a generic point of D over k and let J be the Jacobian variety of C_u defined over $k(u)$. Since the b_j's are rational over k, we can normalize the canonical function φ so that we have $\varphi(b) = 0$ for $b = b_1$ say. We shall denote by M the group of rational points of J with reference to $k(u)$. The group M contains a subgroup N defined in the following way. Let X be a divisor of V which is algebraically equivalent to zero. If X is rational over k, the image point in J of the intersection $X \cdot C_u$ is an element of M. The group N consists of elements of this nature. We note that N can be considered as the group of rational points over k of the Picard variety of V. In particular, it is divisible.[4] Therefore, we have the splitting $M = N \times (M/N)$ and, by Néron's result,[5] the factor group M/N is finitely generated. Moreover, the rank of its free part is equal to $\rho + \beta - 2$. In fact, let V^* be the graph in the product $V \times D$ of the function f. Then V^* is a quadratic transform of V centered at $b_1, \ldots, b_\beta$. In particular, it is non-singular and the Picard number is $\rho + \beta$. Let X^* be a divisor of V^* rational over k such that the intersection $X^* \cdot (C_u \times u) = \Sigma_i n_i (P_i \times u)$ is of degree zero. Then, we have $\varphi(X^*) = \Sigma_i n_i \varphi(P_i) = 0$ if and only if X^* is linearly equivalent on V^* to an integer multiple of $C_b \times b$. Moreover, every element of M can be written in the form $\varphi(X^*)$. Therefore, the rank of the free part of M/N is equal to the Picard number of $V^* - 2$, which is $(\rho + \beta) - 2$ as asserted.

3. *Cohomological Calculation Using Abstract Vanishing Cycle Theory.*[6]—We take a prime number p different from the characteristic of k. Let x be a point of J such that px is in M. We shall denote by L the group of such points. Then, the kernal of the epimorphism of L to M defined by $x \to px$ is the group of points of J of order p, which we shall denote by the standard notation ${}_pJ$. Since ${}_pJ$ is of order p^{2g}, if p does not divide the order of the torsion part of M/N, we have ${}_pJ \cap M = {}_pN$. On the other hand, we know that $k(u)(L)$ is a tamely ramified finite Galois extension of $k(u)$ ramified at $a_1, \ldots, a_\alpha$. Therefore, by a general result of Grothendieck[7] we can state the following lemma:

LEMMA. *The Galois group G of $k(u)(L)$ over $k(u)$ is generated by some generators $\sigma_1, \ldots, \sigma_\alpha$ of inertia groups over $a_1, \ldots, a_\alpha$ satisfying $\sigma_\alpha \ldots \sigma_1 = 1$.*

Now, let v_i be the group of vanishing points which correspond to the inertia group generated by σ_i. It is a cyclic subgroup of ${}_pJ$ of order p and, if x is an arbitrary element of L, we know that $x - \sigma_i x$ is in v_i. Let $(x,y) \to e(x,y)$ be the skew-symmetric non-degenerate bilinear form defined on ${}_pJ$ with values in the cyclic group of pth roots of unity.[8] If p is not in a possible finite set of prime numbers, the induced bilinear form on ${}_pN$ is also non-degenerate. We shall, then, show that an element y of ${}_pJ$ for which $e(x,y) = 1$ for all x in the group generated by ${}_pN$ and $v_1, \ldots, v_\alpha$ is 0, i.e., these subgroups generate the whole ${}_pJ$. Let z be an arbitrary element of ${}_pJ$. Then, we have $e(z - \sigma_i z, y) = 1$ for $i = 1, \ldots, \alpha$, hence $e(z - \sigma z, y) = 1$ for every σ in G. However, this implies $e(z, \sigma^{-1}y - y) = e(\sigma z - z, y) = 1$, hence $\sigma^{-1}y = y$. Therefore, y is in ${}_pJ \cap M = {}_pN$. Since $e(x,y) = 1$ also for all x in ${}_pN$, we get $y = 0$ as asserted. These being remarked, we consider the following exact sequence of G-modules:

$$0 \to {}_pJ \to L \to M \to 0.$$

Then, we get an exact sequence $M \to M \to H^1(G, {}_pJ)$ in which the first homo-

morphism is $x \to px$ and the second one is the connecting homomorphism ∂^*. If we observe that G is generated by $\sigma_1, \ldots, \sigma_\alpha$, we can interpret ∂^* in the following way. Define a homomorphism λ of L to the product $P = v_1 \times \ldots \times v_\alpha$ by

$$x \to (x - \sigma_1 x, \sigma_1 x - \sigma_2 \sigma_1 x, \ldots).$$

Then λ defines a homomorphism λ^* of M to $P/\lambda({}_pJ)$, and this is substantially ∂^*. Therefore $Im(\lambda^*)$ is isomorphic to $M/p(M)$, i.e., to $(M/N)/p(M/N)$. Hence the rank of $Im(\lambda^*)$ is $\rho + \beta - 2$. On the other hand, consider the homomorphism μ of P to ${}_pJ/{}_pN$ defined by

$$(x_1, \ldots, x_\alpha) \to \Sigma_\alpha x_i \bmod {}_pN.$$

We know that μ is surjective. Moreover, since we have $\mu\lambda = 0$, we get an epimorphism μ^* of $P/\lambda({}_pJ)$ to ${}_pJ/{}_pN$. Since the kernel of the restriction of λ to ${}_pJ$ is ${}_pJ \cap M = {}_pN$, which is of rank B_1, the rank of $\lambda({}_pJ)$ is $2g - B_1$. Therefore, the rank of $Ker(\mu^*)$ is $\alpha - 2(2g - B_1)$. Since $Im(\lambda^*)$ is contained in $Ker(\mu^*)$, we finally get

$$\rho + \beta - 2 \leqq \alpha - 2(2g - B_1),$$

and this is another way of writing the inequality $\rho \leqq B_2$.

* Alfred P. Sloan Research Fellow.

[1] Cf. Zariski, O., *Algebraic Surfaces* (New York: Chelsea Publishing Co., 1948).

[2] Cf. Chow, W. L., "On Equivalence Classes of Cycles in an Algebraic Variety," *Ann. Math.*, **64**, 450–479 (1956).

[3] Igusa, J., "Fibre Systems of Jacobian Varieties," *Am. J. Math.*, **78**, 171–199 (1956).

[4] Throughout the paper, we refer to Weil, A., *Variétés Abéliennes et Courbes Algébriques* ["Actualités sci. et ind.," No. 1064 (Paris: Hermann & Cie, 1948)] for results on Abelian varieties.

[5] Néron, A., "Problèmes arithmétiques et géométriques rattachés à la notion du rang d'une courbe algébrique dans un corps," *Bull. Soc. Math. France*, **80**, 101–166 (1952).

[6] Igusa, J., "Fibre Systems of Jacobian Varieties," II, *Am. J. Math.*, **78**, 745–760 (1956); see also Igusa, J., "Abstract vanishing cycle theory," *Proc. Jap. Acad.*, **34**, 589–593 (1958).

[7] Grothendieck, A., "Géométrie formelle et géométrie algébrique," *Sem. Bourbaki* (May 1959).

[8] Weil, A., *op. cit.*, pp. 150–157.

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*NITROGEN FIXATION BY CELL-FREE PREPARATIONS FROM MICROORGANISMS**

BY K. C. SCHNEIDER, CLIVE BRADBEER, R. N. SINGH,† LI CHUAN WANG, P. W. WILSON, AND R. H. BURRIS

DEPARTMENTS OF BIOCHEMISTRY AND BACTERIOLOGY, COLLEGE OF AGRICULTURE, UNIVERSITY OF WISCONSIN

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During the past quarter century, repeated attempts have been made to obtain cell-free nitrogen fixation with extracts from a wide variety of biological agents. Probably the first attempt was that of Bach *et al.*[1] who claimed that cell-free preparations from *Azotobacter chroococcum* fixed sufficient N_2 to be detected by Kjeldahl analysis. However, Roberg[2] could not reproduce these results, and Bach com-

municated to Burk[3] that "The preliminary results[1] . . .await confirmation and extension." In 1943 we reported trials[4] with extracts of *Azotobacter vinelandii* using N^{15} as a tracer which provided a much more sensitive tool for this research. Although these experiments were uniformly unsuccessful, attempts to obtain cell-free fixation were continued with preparations from *Clostridium pasteurianum*, *Rhodospirillum rubrum*, blue-green algae, and leguminous nodules, in addition to the extracts of *A. vinelandii*. Positive results were obtained in a number of experiments,[5-8] but the levels of fixation were usually small and were rarely consistent, so that these studies were of little value in investigating the mechanism of nitrogen fixation. Communications from other laboratories during this period included a report by Wilson and Roberts[9] who also were unable to obtain nitrogen fixation by cell-free extracts of the azotobacter. Nason *et al.*[10] presented a paper indicating that suspensions of *A. vinelandii*, partially disrupted by sonic oscillation, fixed nitrogen at a rate apparently greater than that predicted for the intact cells present. It should be noted that, despite their loss of nitrogen fixing ability, the cell-free preparations from *A. vinelandii* readily utilized ammonia in the synthesis of amino acids and proteins.[11]

In 1958, Hoch and Westlake[12] reported detectable fixation of nitrogen by extracts of *C. pasteurianum*. Although the levels of fixation were small, these results were encouraging, because the preparations appeared to be free from viable cells, and because these tests yielded the first positive results with preparations other than those from the azotobacter. To our knowledge, however, the first report of consistent nitrogen fixation at a high level by cell-free preparations is that of Carnahan, Mortenson, Mower, and Castle.[13] They demonstrated that soluble extracts of *C. pasteurianum*, prepared either by autolysis of dried cells or by rupture of fresh, frozen cells with a Hughes press, gave substantial levels of nitrogen fixation when supplied with pyruvate.

This communication is to report that by employing the preparative techniques of Carnahan *et al.*[13] we have confirmed their significant results and in addition have obtained nitrogen fixation by extracts of *R. rubrum*. Results of cell-free fixation by preparations, obtained by other methods of enzyme extraction, from a number of blue-green algae also are included.

Materials and Methods.—C. pasteurianum was grown at 30°C in a nitrogen-deficient medium;[14] 8 liters, contained in 9-liter serum bottles, was inoculated with 500 ml of an active culture and kept stagnant until growth was initiated, then high purity nitrogen was bubbled slowly through the system. The cultures normally were harvested after about 36 to 48 hours, when the cells were recovered by centrifugation at 2,000 × *g* at 0°C for 15 minutes, washed once in 0.05 *M* phosphate buffer, pH 6.8, and recentrifuged. The cells were resuspended in a small volume of phosphate buffer and were dried under high vacuum at 40° to 50°C with a rotary evaporator. The dried cells were stored under an atmosphere of nitrogen at −10°C until required.

Rhodospirillum rubrum was grown in the light at 30°C in a nitrogen-deficient modification of the medium described by Gest *et al.*[15] The procedures used in the growth of this organism were essentially the same as those for *C. pasteurianum*, and the cells also were harvested and dried in the same way.

The blue-green algae were grown at 24°C in the light in a mineral salts medium

which was the same as that described by Allen and Arnon.[16] Air was bubbled slowly through these cultures.

Cell-free preparations of *C. pasteurianum* and of *R. rubrum* were made from the dried cells by the method of Carnahan *et al.*[13] After suspending the dried cells in a suitable volume of 0.05 *M*, pH 6.8 phosphate buffer (containing 10 mg $Na_2MoO_4 \cdot 2H_2O$ and 10 μg biotin/liter), autolysis was allowed to proceed on a shaker for 1 hour at 30° to 32°C under an atmosphere of hydrogen. The autolysate was subsequently chilled and centrifuged at 20,000 × *g* for 15 minutes at 0°C, and the clear supernatant solution was used directly in the experiments.

Cell-free extracts of the blue-green algae were usually prepared by sonic disruption of the freshly harvested cells in a Raytheon 10 kilocycle magnetostrictive oscillator for 5 minutes at 10°C. The suspensions were centrifuged at 5,000 × *g* at 0°C for 30 minutes; then the supernatant solution was recentrifuged at 20,000 × *g* for 20 minutes. The final supernatant solution was used immediately in the experiments. At times, the supernatant solutions and sedimented pellets obtained by other centrifugal treatments were checked for ability to fix nitrogen.

To test their nitrogen-fixing activity, the cell-free preparations were exposed to atmospheres which contained 0.03 to 0.15 atm N_2 (18 to 96 atom per cent N^{15} excess). The remainder of the atmosphere in the reaction vessels normally consisted of helium in the experiments with the anaerobes and of helium plus oxygen with the blue-green algae. After incubation at 30°C for periods which ranged from 10 minutes to 4 hours, the uptake of N_2^{15} was estimated according to the methods described by Burris and Wilson.[17] We have considered values greater than 0.015 atom per cent N^{15} excess as a conservative indication of positive incorporation of N^{15}. In some experiments the protein in the reaction mixtures was precipitated by 5 per cent trichloroacetic acid, and the TCA-soluble and TCA-insoluble fractions were analyzed separately.

Results.—Clostridium pasteurianum: The results summarized in Table 1 agree with those which have been reported recently by Carnahan *et al.*[13] Sodium

TABLE 1

EFFECT OF PYRUVATE, LACTATE PLUS DPN, AND PHENYL PYRUVATE UPON FIXATION OF NITROGEN BY CELL-FREE EXTRACTS OF *Clostridium pasteurianum*

	Additions			
	Pyruvate, 50 μmoles	Lactate, 100 μmoles; DPN, 27 μmoles	None	Phenyl pyruvate, 100 μmoles
mg N in reaction mixture	1.23	3.99	1.50	1.35
		4.16	1.50	1.26
Atom % N^{15} excess	1.334	0.028	0.192	0.273
		0.033	0.213	0.256

Each flask contained 1 ml enzyme preparation, 5 μmoles α-ketoglutarate, and the additions shown above in a total volume of 2.1 ml; the atmosphere contained 0.15 atm N_2 (96.2 atom % N^{15} excess) and 0.85 atm helium. Alkaline pyrogallol in the center wells absorbed O_2; incubation was at 32°C for 3 hours.

pyruvate proved to be the most effective substrate in promoting the fixation of nitrogen; the precise role of pyruvate in the process is not known. The fixation obtained in the absence of added pyruvate may have been supported by endogenous pyruvate present in the enzyme preparation. As shown by Carnahan *et al.*,[13] α-ketoglutarate was ineffective in supporting nitrogen fixation, but was routinely added to the reaction mixtures in our experiments to prevent the accumulation of possibly inhibitory levels of ammonia. Lactate plus DPN was unable

TABLE 2

TIME COURSE OF NITROGEN FIXATION BY CELL-FREE EXTRACTS FROM *Clostridium pasteurianum*

Experiment A			Experiment B		
Time in Minutes	mg N per Vessel	Atom % N^{15} Excess	Time in Minutes	mg N per Vessel	Atom % N^{15} Excess
10	2.67	0.011		1.96	0.720
			30	1.99	0.978
				1.52	0.862
20	2.67	0.097			
				1.81	1.466
			60	1.91	1.322
				1.96	1.576
40	2.61	0.134			
	2.65	0.244			
				2.17	1.008
			120	2.11	1.129
				1.35	1.289
160	2.71	0.707			
				1.94	1.190
			240	1.94	1.312
				2.24	0.938

Experiment A. Each flask contained 2 ml enzyme preparation, 20 mg sodium pyruvate, and 5 μmoles α-ketoglutarate in a total volume of 2.6 ml; alkaline pyrogallol was in the center well to absorb traces of oxygen. Gas phase consisted of 0.04 atm N_2 (96.2 atom % N^{15} excess) and 0.96 atm helium. After the indicated time intervals, 1 ml of 3 *N* H_2SO_4 in the side arms was tipped into the main compartment to stop the reaction.

Experiment B. Each flask contained 1 ml enzyme preparation, 10 mg sodium pyruvate, and 5 μmoles α-ketoglutarate in a total volume of 1.5 ml; alkaline pyrogallol was in the center well. The gas phase consisted of 0.13 atm N_2 (96.2 atom % N^{15} excess) and helium to one atmosphere.

to substitute for pyruvate in supporting nitrogen fixation and instead proved to be inhibitory. The inclusion of phenyl pyruvate in the reaction mixtures gave levels of fixation which were only slightly higher than those obtained in the controls without pyruvate.

Table 2 shows the results of two experiments on the time course of nitrogen fixation by cell-free preparations. In experiment A, in which each flask contained 20 mg of sodium pyruvate, the fixation of nitrogen continued for 160 minutes and the highest N^{15} concentration was in the last sample taken. However, in experiment B, in which each flask contained only 10 mg of pyruvate, the maximum levels of fixation were obtained at 60 minutes. It is not clear why the 120-minute and 240-minute values in experiment B were lower than those obtained after 60 minutes. It is possible that the manifolds bearing these flasks may have leaked, admitting air which reduced the effective atom per cent N^{15} excess of the nitrogen.

TABLE 3

ISOLATION OF FREE AMMONIA, GLUTAMINE AMIDE N, AND ASPARAGINE AMIDE N FROM THE REACTION MIXTURES AFTER CELL-FREE FIXATION OF NITROGEN BY EXTRACTS OF *Clostridium pasteurianum*

Time in Minutes	N Fraction	mg N	Atom % N^{15} Excess
60	Ammonia	0.070	42.32
	Glutamine amide N	0.120	14.2
	Asparagine amide N	0.185	0.809
180	Ammonia	0.191	52.06
	Glutamine amide N	0.122	8.666
	Asparagine amide N	0.345	0.569

Eight flasks were set up, each containing 1 ml enzyme preparation and 20 mg sodium pyruvate in a total volume of 1.2 ml. The center wells contained alkaline pyrogallol. The gas phase consisted of 0.10 atm N_2 (96.2 atom % N^{15} excess) and 0.90 atm helium. After 60 minutes incubation (32°C), the contents of four flasks were harvested and pooled and subsequently analyzed. After 180 minutes the procedure was repeated with the remaining four flasks.

Table 3 furnishes some results from an experiment which was designed to investi-

gate the products of N_2^{15} fixation by the cell-free extracts. After allowing fixation of nitrogen (containing 96.2 atom per cent N^{15} excess) to proceed for periods of one and three hours, various nitrogenous fractions in the reaction mixtures were isolated and analyzed for N^{15}. The free ammonia was separated from the reaction mixtures by distillation *in vacuo* at 40°C and pH 10 according to the method of Pucher *et al.*[18] Subsequently, the pH of the reaction mixtures was adjusted to 6.5 and glutamine was hydrolyzed at 120°C in the autoclave for 30 minutes. The resultant ammonia, which represented the glutamine amide nitrogen, was obtained by distillation *in vacuo* at pH 10 as before. The reaction mixtures then were acidified to give a final concentration of *N* H_2SO_4, and asparagine was hydrolyzed in the autoclave for one hour. The ammonia, which represented the asparagine amide nitrogen was once more obtained by distillation at pH 10. These methods for glutamine and asparagine amide nitrogen are essentially the same as those described by Vickery *et al.*[19] Values of 42 and 52 atom per cent N^{15} excess were found in the free ammonia (see Table 3). The amide nitrogen of glutamine contained substantial, but lower, amounts of N^{15}, and the atom per cent N^{15} excess of the asparagine amide nitrogen was relatively low. These observations support the ammonia hypothesis of nitrogen fixation. The high levels of N^{15} in the free ammonia increase the likelihood that, with these cell-free preparations, the intermediates between molecular nitrogen and ammonia may be identified.

Rhodospirillum rubrum: The results of some preliminary experiments on nitrogen fixation by cell-free extracts of *R. rubrum* are given in Table 4. Although the levels

TABLE 4

NITROGEN FIXATION BY CELL-FREE EXTRACTS OF *Rhodospirillum rubrum*

Experiment		Light Treatment	mg N per Vessel	Atom % N^{15} Excess
1	10 mg pyruvate	light	1.1	1.172, 0.537
	10 mg pyruvate	dark	1.2	1.231, 0.726
2	None	light	0.2	0.796, 1.15
	None	dark	0.2	1.040, 0.848
3	10 mg α-ketoglutarate	dark	0.3	1.586
	10 mg α-ketoglutarate	light	0.2	3.082
	5 mg pyruvate + 10 mg α-ketoglutarate	light	0.2	2.53
	5 mg pyruvate + 10 mg oxalacetate	light	0.2	2.14
	10 mg oxalacetate	light	0.2	1.212
	10 mg pyruvate + 1 μg TPN	light	0.2	1.095
	10 mg pyruvate + 350 μg DPNH	light	0.2	1.020
	None	light	0.2	1.070

The reaction mixtures usually contained 2 ml of the enzyme preparation, together with the indicated additions, in a total volume of about 2.5 ml. The gas phase usually contained 0.06 atm N_2 (96.2 atom % N^{15} excess) and 0.5 atm of helium. Incubation was for two hours at 30°C.

of fixation are high, these experiments give no clear-cut indication of the source of the reducing power necessary for the reduction of the elemental nitrogen. Even in the absence of added substrates, the fixation levels in the dark were approximately the same as those obtained in the light. Thus, these enzyme preparations seem to have sufficient endogenous "reducing power" to support very satisfactory levels of nitrogen fixation. Experiments are at present in progress to identify this "reducing power" and to investigate its precise relationship to the nitrogen fixation process.

Blue-green algae: The results of some experiments on N_2^{15} fixation by cell-free extracts of five species of blue-green algae are shown in Table 5. These experiments

TABLE 5

FIXATION OF NITROGEN BY CELL-FREE EXTRACTS OF FIVE SPECIES OF BLUE-GREEN ALGAE

Exp.	Organism	Centrifugal Treatment	Period of Incubation	Atom % N^{15} Excess
1	*Mastigocladus laminosus*	2,000 × *g*, 30 min	60 min	0.025, 0.015
2	*Nostoc muscorum G*	2,000 × *g* 20 min	90 min	0.018, 0.040
	Anabaena cylindrica	2,000 × *g*, 20 min	90 min	0.016, 0.037
	Calothrix parietina	2,000 × *g*, 20 min	90 min	0.026, 0.049
	M. laminosus	2,000 × *g*, 20 min	90 min	0.392, 0.240
3	*M. laminosus*	Whole cells	90 min	0.063, 0.247
		2,000 × *g*	90 min	0.037, 0.017
		20,000 × *g*	90 min	0.019, 0.035
4	*M. laminosus*	45,000 × *g*	135 min	0.028, 0.013
5	*Gloeotrichia echinulata*	5,000 × *g*	120 min	0.012, 0.036
		45,000 × *g*	120 min	0.010, 0.014

The atom per cent N^{15} excess values in experiments 1, 2, and 3 are for the TCA-soluble fraction of the reaction mixtures. In experiments 4 and 5 the whole reaction mixtures were analyzed for N^{15}. The enzyme preparations used consisted of the supernatants which remained after the indicated centrifugal treatments. The gas phases used were:

Experiment 1 — 0.1 atm N_2 (96 atom % N^{15} excess) 0.2 atm O_2.
2 — 0.1 atm N_2 (96 atom % N^{15} excess) 0.3 atm O_2.
3 — 0.1 atm N_2 (60 atom % N^{15} excess) 0.2 atm O_2.
4 — 0.2 atm N_2 (18 atom % N^{15} excess).
5 — 0.2 atm N_2 (18 atom % N^{15} excess).

The remainder of the gas phase always was helium.

TABLE 6

FIXATION OF NITROGEN BY CELL-FREE EXTRACTS OF *Mastigocladus laminosus* and *Gloeotrichia echinulata*

Exp.	Organism	Incubation Conditions	Additions	Atom % N^{15} Excess	mg N/Sample
6	*M. laminosus*	light	none	0.036, 0.059	....
	M. laminosus	light	ATP, DPN, TPN	0.105, 0.029	....
	M. laminosus	light	ADP, yeast conc.	0.054, 0.013	....
	G. echinulata	light	none	0.049, 0.119	....
	G. echinulata	light	ATP	0.074, 0.023	....
	G. echinulata	light	yeast conc.	0.086, 0.014	....
7a	*M. laminosus*	light	none	0.115, 0.293	0.182, 0.141
		light	ATP	0.088	0.263
		dark	none	0.181, 0.024	0.187
7b	*M. laminosus*	light	none	0.143, 0.103	0.370
		anaerobic dark	none	0.137, 0.164	0.402, 0.357
		anaerobic light	none	0.183, 0.360	0.325, 0.347
8	*M. laminosus*	anaerobic light	none	0.341, 0.138	0.685, 0.685
		anaerobic light	ATP	0.291, 0.328	0.760, 0.760

The cells were broken with a sonic oscillator except for experiment 7a in which the Hughes press was used. In experiment 6, the broken cells were centrifuged at 5,000 × *g* for 30 min and in experiments 7 and 8 at 12,000 × *g* for 30 minutes. The supernatant solution was used as the source of the enzyme; ATP (10 μmoles), ADP (10 μmoles), DPN (2 mg), TPN (2 mg), and Sigma yeast concentrates II and IV (0.5 mg) were added as indicated. The gas phase in all flasks contained 0.1 atm N_2 (96.2 atom % N^{15} excess). Except where indicated 0.1 to 0.2 atm O_2 was also included and the remainder consisted of helium. The reaction mixture was exposed to N_2^{15} at 30 to 32°C for 120 min.

were all performed in the light at 28° to 30°C. The nitrogen-fixing activity was not sedimented from suspensions of the broken cells by centrifugal forces up to 45,000 × *g* for 45 minutes. Since the most active preparations were regularly obtained from *Mastigocladus laminosus*, this organism was used most frequently in subsequent experiments.

Table 6 shows the results of some changes in the reaction conditions in experiments with *M. laminosus* and *Gloeotrichia echinulata*. The N^{15} levels obtained in

the light tended to be somewhat higher than those obtained in the dark and anaerobic conditions seemed to be just as favorable as aerobic. The incorporation of ATP, DPN, and TPN in the reaction mixtures had no consistent effect on the levels of nitrogen fixation.

Discussion.—The discovery of Carnahan and co-workers[13] that dried preparations of *C. pasteurianum* can yield soluble enzyme systems capable of nitrogen fixation should prove of great value in studies on the mechanism of the nitrogen fixation process. Our success in applying their method to *Rhodospirillum rubrum* suggests that the mode of preparation of active extracts may be generally successful for anaerobic nitrogen fixers. As yet, these methods have not been applied successfully to aerobic organisms.

The blue-green algae also show promise for studies of cell-free nitrogen fixation. Although the fixation to date has been less vigorous than that obtained with the anaerobes, it consistently has been at a level which can be studied easily. It will be of particular interest to determine whether or not there is a specific coupling between the photosynthetic reaction and biological nitrogen fixation.

The availability of consistently active cell-free, nitrogen-fixing preparations should hasten the discovery of the intermediates in this process. Our preliminary studies, i.e., the isolation of ammonia with a high level of N^{15} from the reaction mixture, and those of Carnahan *et al.*[13] already have verified the position of ammonia as an early product of nitrogen fixation. No convincing evidence has yet been obtained to identify the intermediates occurring between molecular nitrogen and ammonia, but the isolation of these products now seems much more feasible. Since fixation of nitrogen by the extracts of *C. pasteurianum* appears to be coupled to the metabolism of pyruvate, it will be of interest to investigate this relationship more completely.

Summary.—Cell-free nitrogen fixation has been achieved with preparations from *Clostridium pasteurianum*, *Rhodospirillum rubrum*, and several species of blue-green algae. The observations of Carnahan *et al.*[13] with *C. pasteurianum* have been fully verified. The fixation by the preparations from this organism is coupled to the metabolism of pyruvate. The isolation of free ammonia, containing up to 52 atom per cent N^{15} excess, from the reaction mixtures provides support for the ammonia hypothesis of nitrogen fixation.

This communication appears to be the first report of substantial cell-free fixation of nitrogen by *Rhodospirillum rubrum* and by blue-green algae.

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[1] Bach, A. N., Z. V. Jermolieva, and M. P. Stepanian, *Compt. rend acad. sci.* (USSR), **1**, 22 (1934).

[2] Roberg, M., *Jahrb f. wiss. Bot.*, **83**, 567 (1936).

[3] Burk, D., *Biokhimiya*, **2**, 312 (1937).

[4] Burris, R. H., F. J. Eppling, H. B. Wahlin, and P. W. Wilson, *J. Biol. Chem.*, **148**, 349 (1943).

[5] Hamilton, P. G., W. E. Magee, and L. E. Mortenson, *Bact. Proc.*, 82 (1953).

[6] Burris, R. H., in *Inorganic Nitrogen Metabolism*, ed. W. D. McElroy and B. Glass (Baltimore: Johns Hopkins Press, 1956), p. 316.

[7] Magee, W. E., and R. H. Burris, *J. Bact.*, **71**, 635 (1956).

[8] Temperli, A., and P. W. Wilson, *Experientia*, **14**, 363 (1958).

[9] Wilson, T. G. G., and E. R. Roberts, *Nature*, **174**, 795 (1954).

[10] Nason, A., H. Takahashi, G. Hoch, and R. H. Burris, *Fed. Proc.*, **16**, 224 (1957).

[11] Burma, D. P., and R. H. Burris, *J. Biol. Chem.*, **225**, 723 (1957).

[12] Hoch, G. E., and D. W. S. Westlake, *Fed. Proc.*, **17**, 243 (1958).

[13] Carnahan, J. E., L. E. Mortenson, H. F. Mower, and J. E. Castle, *Biochim. Biophys. Acta*, **38**, 188 (1960).

[14] Westlake, D. W. S., and P. W. Wilson, *Can. J. Microbiol.*, **5**, 617 (1959). The medium contained per liter: 20 gm sucrose, 15.6 gm K_2HPO_4, 1.4 gm KH_2PO_4, 50 mg anhydrous $MgSO_4$, 25 mg $CaCl_2 \cdot 2H_2O$, 2 μg biotin, 60 mg Difco yeast extract, 3 mg Fe as $FeSO_4$, 1.5 mg Mo as Na_2MoO_4, and 0.1 mg Mn as $MnSO_4$.

[15] Gest, H., M. D. Kamen, and H. M. Bregoff, *J. Biol. Chem.*, **182**, 153 (1950). The medium contained per liter: 200 mg $MgSO_4 \cdot 7H_2O$, 50 mg $CaCl_2 \cdot 2H_2O$, 500 mg KH_2PO_4, 500 mg $Na_2HPO_4 \cdot 2H_2O$, 4 gm DL malic acid, 1 μg biotin, 25 mg Difco yeast extract, 20 mg $(NH_4)_2SO_4$, 3 mg Fe as $FeSO_4$, and 1 mg Mo as Na_2MoO_4; the medium was adjusted to pH 6.7 with NaOH.

[16] Allen, M. B., and D. I. Arnon, *Plant Physiol.*, **30**, 366 (1955).

[17] Burris, R. H., and P. W. Wilson, in *Methods in Enzymology, IV*, ed. S. P. Colowick and N. O. Kaplan (New York: Academic Press Inc., 1957), p. 355.

[18] Pucher, G. W., H. B. Vickery, and C. S. Leavenworth, *Ind. Eng. Chem., Anal. Ed.*, **7**, 152 (1935).

[19] Vickery, H. B., G. W. Pucher, H. E. Clark, A. C. Chibnall, and R. G. Westall, *Biochem. J.*, **29**, 2710 (1935).

THE INHIBITION OF NUCLEIC ACID AND PROTEIN SYNTHESIS IN TRYPANOSOMA LEWISI BY THE ANTIBODY ABLASTIN, †*

BY WILLIAM H. TALIAFERRO AND TULIO PIZZI‡

DEPARTMENT OF MICROBIOLOGY, UNIVERSITY OF CHICAGO

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The early microscopical observations of Rabinowitsch and Kempner[1] in 1899 established the fact that *Trypanosoma lewisi* reproduce in the blood of rats only during the first few days of the infection and remain thereafter in the blood for several weeks or months as nonreproducing adults.[2] In 1924, Taliaferro[3] demonstrated that this behavior is brought about by the development in the rat of a passively transferable serum component which specifically inhibits mitosis and growth of the parasites without detectable effects on their vitality or infectivity. Later, he named the serum component ablastin and showed that it is a globulin and possesses the other general characteristics of an antibody except that it is not adsorbed from immune serum by living parasites.[4] The characteristic of nonadsorbability may be the result of an unsatisfactory antigen preparation and/or a very low avidity, i.e., a low antigen-binding capacity.[5] It has proved to be very useful for separating ablastin from the trypanocidal factors in serum.

Using the term trypanocidal to include all serum elements which when injected into infected rats decrease the number of circulating parasites, Coventry[6] described a trypanocidal antibody that kills the parasites early in the infection and gives rise to the first parasite crisis and a second trypanocidal antibody that acts on the surviving parasites to terminate the infection. The second antibody can be irregularly associated with a trypanolysin.[4] Augustine[7] found that the early trypanocidal antibody acted chiefly on the dividing parasites by immobilizing them and making them susceptible to phagocytosis. This antibody (or a second one), at high titer, agglutinated the adult parasites and thus facilitated their mechanical removal and destruction by the liver and probably the spleen. Barnes[8] described a functional agglutinin as well as ablastin and a trypanolysin. At times parasites disappear gradually.[9] Recently, D'Alesandro[10] found that the early trypanocidal antibody and ablastin are of relatively small molecular size with a sedimentation constant of 6 Svedbergs, whereas the late trypanocidal antibody is a larger molecule with a sedimentation constant of 16 Svedbergs. All localize between the slow-moving beta globulins and the fast-moving gamma globulins during starch block electrophoresis.

Several other interpretations have been advanced to explain the exact mechanism by which the parasites are destroyed. Thus, Regendanz and Kikuth[11] associated the disappearance of trypanosomes from the blood with nonspecific phagocytosis after ablastin had inhibited reproduction. Thillet and Chandler[12] postulated that ablastin inhibits reproduction when low in titer and agglutinates and renders the trypanosomes more susceptible to phagocytosis in high concentrations.

Thillet and Chandler[12] also reported that ablastin can be adsorbed with metabolic products, i.e., soluble antigens which diffuse from the trypanosomes when they are incubated in serum overnight. We do not question the possibility of finding experimental conditions under which ablastin can be adsorbed. Nevertheless, we cannot accept their conclusion since no direct evidence of adsorption was reported. They prepared an immune serum with protective activity which they assumed, but did not demonstrate, to possess ablastic activity. Thus, low doses of this serum gave no evidence of ablastic activity and high doses were so trypanocidal that according to their own statement the parasites were killed before ablastic action could be seen. Furthermore, Taliaferro[4] and D'Alesandro[10] found that absorbing immune serum with living parasites does not lower the ablastic titer, but selectively removes all factors which reduce the number of parasites *in vivo* whether by killing or by temporarily removing them from the peripheral blood.

Ablastin-induced inhibition of reproduction causes the rapid disappearance of division in the nucleus as well as the duplication of other organelles and the gradual disappearance of variability associated with division and growth. Thus, an inhibited population frequently shows the remarkably low coefficient of variation for total length (including the free flagellum) of about 3 per cent.[13] In blood smears stained with Romanowsky stains, the cytoplasm of dividing parasites is markedly basophilic, while that of inhibited forms is much more eosinophilic. This finding suggests an inhibition of nucleic acid and protein synthesis.

The present work is a study of nucleic acid and protein synthesis as modified by ablastin. Biochemically, it is an extension of work by Moulder[14] showing

that changes in the glucose metabolism of *T. lewisi* can be correlated with the inhibition of reproduction by ablastin during the course of infection. He found that dividing parasites consume less oxygen but utilize more glucose per parasite than adults and that their respiratory quotient and the ratio of oxygen consumption to glucose consumption are lower than in adults. He suggested that ablastin shifts the metabolism of the parasites from an active oxidative assimilation to one of maintenance. These findings have been confirmed by later workers.[15]

Materials and Methods.—Materials and methods employed in the present study are considered in greater detail elsewhere.[16]

All infections were produced in Holzman female rats with a strain of *T. lewisi* that has been maintained by rat passages many years and is the same one used by Moulder.[17] Organisms can be found in blood smears made within an hour after the intraperitoneal injection of a large number of parasites. The relative amount of reproduction and growth can be determined by the per cent of division forms, i.e., parasites showing evidences of division in the cell organelles *plus* the small and large forms associated with division.[18] Reproduction is very active during the first three days when rats are injected with dividing parasites, but may be initially delayed up to a day when started with adult parasites. Division forms are rarely observed after day 5 and never after day 7 in the infections used in the present work (Figs. 1, 2, and 4). Released *T. lewisi* refer to adult trypanosomes which after having been taken from a rat infected 6 or more days had been washed free of ablastic serum and had been injected 3 hours previously into an uninfected rat.

The S^{35}-amino acids (chiefly methionine with small amounts of cystine) consisted of an acid hydrolysate of yeast to which tryptophane was added in approximately the amount lost by acid hydrolysis. It was prepared by Abbott Laboratories. Adenine-8-C^{14} was prepared by the Schwartz Laboratories and the Nuclear-Chicago Corporation. With few exceptions both compounds were given intraperitoneally in doses of 0.01 mc per 100 gm rat.

The basic medium for various manipulations of the trypanosomes *in vitro* was a calcium-free phosphate-saline solution (pH 7.4–7.6) containing 0.01 *M* glucose and 2 per cent bovine serum albumin as described by Moulder.[17] Trypanosomes were obtained by drawing blood from the heart of an infected anesthetized rat into a syringe containing 1 ml of 10 per cent sodium citrate. The citrated blood was mixed with equal amounts of cold phosphate saline containing rabbit antirat hemagglutinin.[17] The hemagglutinin not only aided in separating the trypanosomes from the red cells by differential centrifugation but also precipitated platelets and probably other sources of nonspecific radioactivity.

The trypanosomes were then prepared for radioassay as follows. The parasite suspension was mixed with an equal amount of freshly prepared cold 20 per cent trichloroacetic acid (TCA) plus 0.2 per cent of the carrier compound corresponding to the particular compound used (methionine and cystine or adenine). After 10 minutes on ice, the resulting precipitate was separated from the supernatant fluid by centrifugation in the cold. This procedure was repeated twice and, finally, the TCA precipitate was dissolved in 0.04 *N* NaOH for plating and counting on dried oxidized copper planchets (area of 10 cm^2).

Although all counts shown in the figures and tables were made on the combined

protein and nucleic acid fraction precipitated by cold TCA, we consider S^{35} from labeled amino acids to be solely in the proteins and C^{14} from labeled adenine to be solely in the nucleic acids. This division was made on the basis of extracting the nucleic acids from some of the cold-TCA precipitates with hot TCA according to the method of Schneider.[19] This method demonstrated that S^{35} was in the fraction insoluble in both cold and hot TCA, i.e., the proteins, whereas C^{14} was in the cold-TCA insoluble and hot-TCA soluble fraction, i.e., the nucleic acids.[16]

All specific radioactivity is expressed in counts (cpm) per minute per million parasites suitably corrected for size on day 1 and 2 when the infections were initiated with dividing parasites and on day 2 when they were initiated with adult organisms.[16] Specific radioactivity was related to μg protein N by the regression equation:

$$\text{cpm per } \mu\text{g protein N} = 84.7 + 2.2 \times \text{cpm per } 10^6 \text{ trypanosomes.}$$

Radioassays were made in a gas flow internal Geiger counter (model 200A, Packard Co.). When necessary, corrections were made for resolving time, self absorption, and, with the S^{35} preparations, for decay. Background counts were approximately 40 counts per minute. In most cases, the total count for individual preparations was over 20,000 with a relative standard error of the difference < 0.01.

Experimental Results.—Protein synthesis: The effect of ablastin on protein synthesis was studied by using S^{35} in the following 4 ways. (1) Rats were given one or repeated injections of S^{35}-amino acids on various days throughout developing infections and were killed 2 or 3 hours after various doses of the isotope to obtain trypanosomes for radioassay. Comparison of the rate of incorporation of S^{35} with the per cent of division forms among the parasites would thus indicate the effectiveness of ablastin as it is formed naturally by the host in response to the infection. (2) Rats with 3-day and with 8- to 22-day infections were injected with S^{35} and were killed from 15 minutes to 48 hours later to obtain samples of trypanosomes for radioassay to ascertain the difference in protein synthesis in dividing trypanosomes and in adults inhibited by ablastin. (3) The incorporation of S^{35} into protein by trypanosomes obtained from rats at different stages of the infection was studied in *in vitro* preparations. (4) Ablastin was passively transferred to rats 1 day after infection with dividing trypanosomes and 2 days before injection of S^{35} to study protein synthesis in relation to the action of passively transferred ablastin on dividing *T. lewisi*.

Figure 1 shows the changes in the rate of incorporation of S^{35} into protein from amino acids as the infection progressed for 19 days and as reproduction of the trypanosomes was being inhibited. One injection of the S^{35} amino acids was given at various intervals during the infection to groups of rats, and the rats were killed 2 or 3 hours later to obtain trypanosomes for radioassay. The highest rate of incorporation (198 cpm) occurred in the released trypanosomes from 3-hour infections in 3 rats given isotope 3 hours before. The parasites were removed from 8-day infections and were washed once to remove the donor rat's immune serum. They were then injected into the 3 experimental rats just before the latter received 0.01 mc S^{35}-amino acids per 100 gm. and were removed from the experimental rats for radioassay 3 hours later. Morphologically, the trypanosomes were adults except

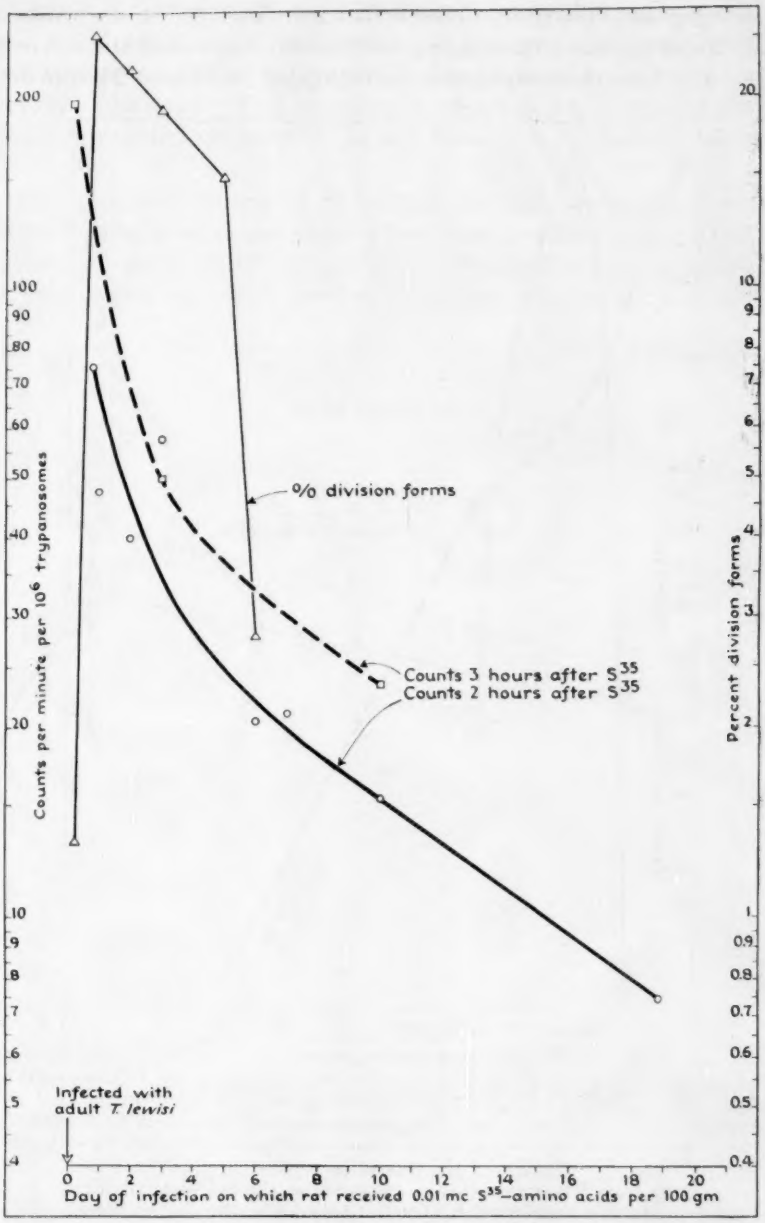
Counts per minute per 10^6 trypanosomes
Percent division forms
% division forms
Counts 3 hours after S^{35}
Counts 2 hours after S^{35}
Infected with adult *T. lewisi*
Day of infection on which rat received 0.01 mc S^{35}—amino acids per 100 gm

FIG. 1.—Changes in the rate of incorporation of S^{35} into protein from S^{35}-amino acids as compared to the rise and fall of reproduction (per cent division forms) among the trypanosomes during 19 days of the infection. Trypanosomes for radioassay were taken 2 hours (heavy solid line) or 3 hours (heavy broken line) after a single dose of 0.01 mc of S^{35} per 100 gm rat administered to 88 rats at different stages of their infection. Each point is a mean derived from 3 to 23 similarly treated infections.

that 1.5 per cent were slightly larger. Thereafter, the rate of incorporation of S^{35} greatly decreased. It showed a mean of 58 and 50 cpm for the 2- and 3-hour observations, respectively, at 3 days. Because at this time, the trypanosomes were

rapidly dividing and, therefore, ablastin had not developed, these values will be considered the zero point in evaluating the inhibitory action of ablastin on protein synthesis. The rate of incorporation had dropped to 15 and 24 cpm for the 2

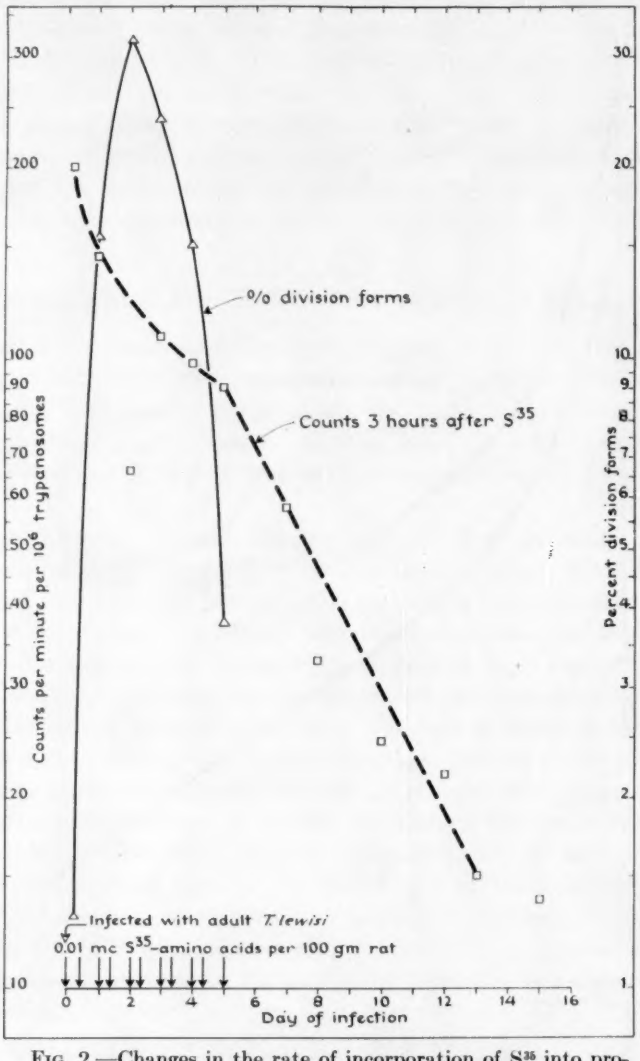

FIG. 2.—Changes in the rate of incorporation of S^{35} into protein from S^{35}-amino acids as compared to the rise and fall of reproduction (per cent division forms) among the trypanosomes during and after the administration of multiple doses of 0.01 mc S^{35} per 100 gm rat to 39 rats. During the period that the isotope was being given, trypanosomes for radioassay were obtained 3 hours after a given dose. Each point is a mean obtaned from 2 to 6 similarly treated infections.

mean observations on day 10. These values represent an inhibition of 74 and 52 per cent, respectively, for the assays made 2 and 3 hours, respectively, after administration of the isotope. It should be noted in calculating these percentages that the actual reading of 58 ± 5 was used for the 3-day 2-hour mean because

it was based on 22 determinations. The 3-day 2-hour fitted value from the graph (34 cpm) would result in an inhibition of 56 per cent. After day 10, the rate continued to decrease slowly until on day 19, the 2-hour value of 7 cpm represented an 88 per cent inhibition. That synthesis decreased slowly is indicated by the fact that the differences in incorporation for day 8 and day 19 showed a probability of 0.05.

The rapid protein synthesis by morphologically appearing adults, which have been released from ablastin inhibition a few hours previously, is further corroborated by the very rapid loss of radioactivity in S^{35}-labeled adult trypanosomes after transfer to a normal rat which received no isotope. Under these conditions, the

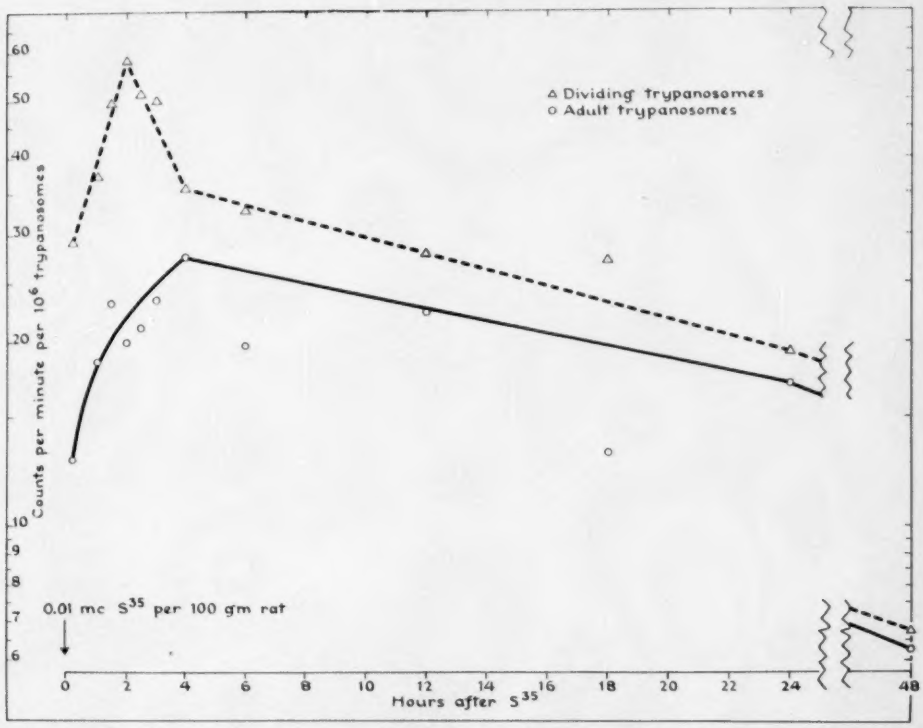

FIG. 3.—The rise and fall of specific radioactivity of trypanosomal protein following a single injection of 0.01 mc S^{35} into 80 rats containing 3-day dividing trypanosomes (broken line) and 83 rats with 6- to 21-day infections consisting of adults (solid line). Each point is a mean derived from 2 to 24 similarly treated infections.

radioactivity disappeared very rapidly for about 4 hours and then disappeared more slowly with a half disappearance time of 7.2 hours.

A study was then made of multiple injections of S^{35} in 14 rats during 15 days after the injection of adult trypanosomes from 8-day infections. The first dose of 0.01 mc of S^{35} was injected immediately after the trypanosomes were injected. This dose was followed by similar amounts injected twice daily at 8:30 A.M. and 4:30 P.M. until a total of 0.11 mc S^{35} had been administered. Trypanosomes were obtained for radioassay daily 3 hours after the morning injection. The data are shown in Figure 2. The first determination (198 cpm) is a mean from the same

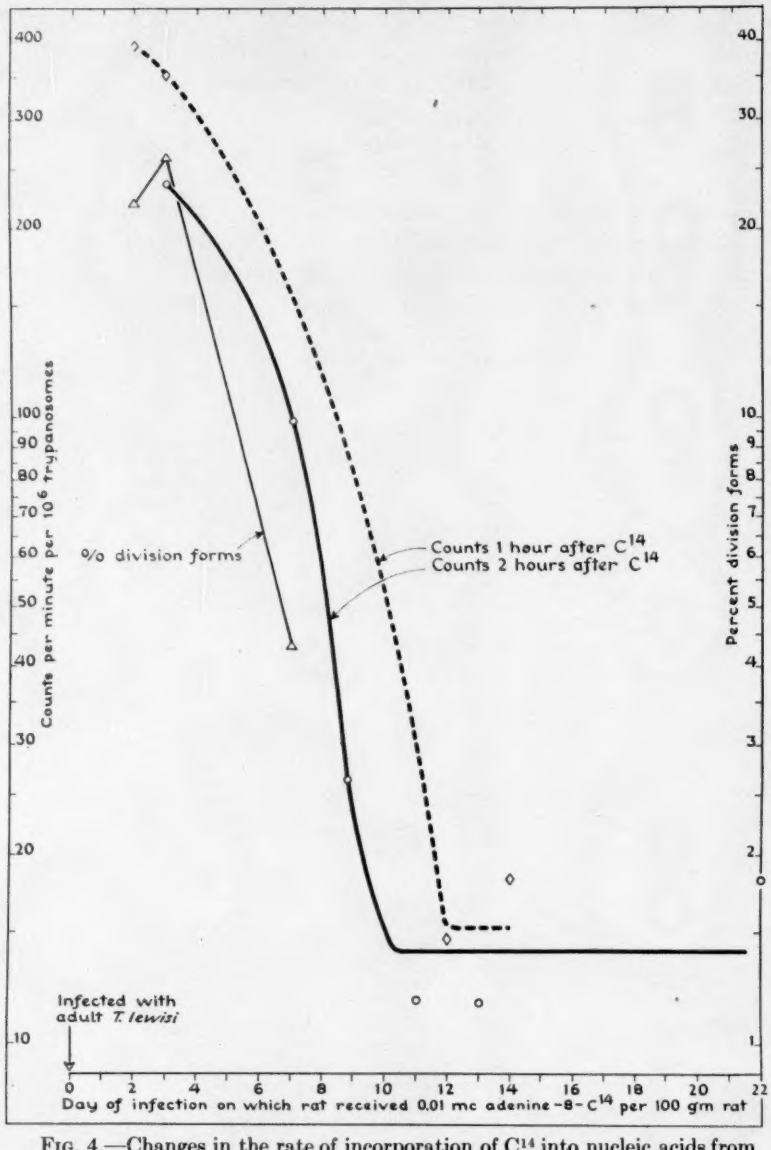
Counts per minute per 10^6 trypanosomes
Percent division forms
% division forms
Counts 1 hour after C^{14}
Counts 2 hours after C^{14}
Infected with adult *T. lewisi*
Day of infection on which rat received 0.01 mc adenine-8-C^{14} per 100 gm rat

FIG. 4.—Changes in the rate of incorporation of C^{14} into nucleic acids from adenine-8-C^{14} as compared to the rise and fall of reproduction (per cent division forms) among the trypanosomes during 22 days of the infection. Trypanosomes for radioassay were taken 1 hour (heavy broken line) or 2 hours (heavy solid line) after a single dose of 0.01 mc of adenine-8-C^{14} per 100 gm rat administered to 48 rats at different stages of their infection. Each point is a mean obtained from 2 to 12 similarly treated infections.

group of rats as in Figure 1. Thereafter, while the isotope was being given, the specific radioactivity of the parasites decreased rapidly although not as rapidly as when only one dose of S^{35} was given. Thus, on day 5, it was 37 cpm in Figure 1 and 92 cpm in Figure 2. The slower decline during the first 5 days in Figure 2 as compared to Figure 1 undoubtedly resulted from the greater availability of the isotope

TABLE 1

INCORPORATION OF S^{35} *in vitro* INTO PROTEIN OF *Trypanosoma lewisi* WHICH WERE REMOVED FROM RATS AT DIFFERENT STAGES OF THE INFECTION

Day of infection	S^{35}-amino acids in 5 ml	Incubation time at 37°C	Counts per minute per 10^6 trypanosomes	Per cent inhibition
3	0.1 μc	1 hour	98	*
4	0.1 μc	1 hour	25	74
8	0.1 μc	1 hour	21	79
3	1.0 μc	2 hours	226	*
9	1.0 μc	2 hours	157	31

* Considered zero inhibition at 1 and 2 hours, respectively.

because of the increased dosage and recycling of S^{35}-amino acids. After administration of the isotope was stopped at 5 days, the parasites, which by this time were adults, lost radioactivity at an apparent half life of about 3.1 days.

The relative rate of incorporation of amino acids into proteins was then compared in dividing and in adult, i.e., ablastin-inhibited, trypanosomes. For this series of experiments, a single dose of 0.01 mc S^{35} per 100 gm. rat was given (1) to rats with 3-day infections in which the parasites were still undergoing division and growth (20 per cent division forms as noted in Figure 1) and (2) to rats with 6- to 12-day infections in which there was no microscopic evidence of division or growth. Trypanosomes for radioassay were obtained from these rats at various intervals from 20 minutes to 48 hours after the injection of S^{35}. The results are plotted in Figure 3. The dividing parasites incorporated S^{35} into their protein fraction at a rapid and uniform rate for 2 hours. At this time, a peak of 58 ± 5 cpm was reached. In contrast, adults incorporated the isotope at a less rapid and decreasing rate for 4 hours. The peak was also lower, i.e., 28 ± 2 cpm. The rapid loss of specific radioactivity between 2 and 4 hours in the dividing parasites was undoubtedly in part a dilution effect arising from the fact that the S^{35} incorporated during the preceding 2-hour period was being rapidly distributed to an increasing amount of trypanosome protein as the population increased. During the succeeding 44 hours between hours 4 and 48, radioactivity was lost faster by 24 per cent in the dividing forms ($t^1/_2$ = 22 hours) than in the adults ($t^1/_2$ = 29 hours). The lower rate of protein synthesis in adults raises the question as to why the dividing parasites did not lose their radioactivity from 4 hours on at a much faster rate than the adults. We believe that the dividing forms were rapidly becoming adult. Thus, the trypanosomes which were dividing when the isotope was administered on day 3 were largely adult by day 4.

In vitro determinations corroborated the *in vivo* findings on the incorporation of S^{35}. Thus, as shown in Table 1, a count of 98 cpm was reached for 3-day parasites after a 1-hour incubation with 0.0001 mc S^{35}-labeled amino acids. This count decreased by 74 per cent for 4-day parasites and by 79 per cent for 8-day parasites. The marked inhibition at 4 days is consistent with the results shown in Figure 1. Similar results were obtained with 0.001 mc S^{35} and a 2-hour incubation, but the inhibition was less. Longer *in vitro* intervals were not tested because from 10 to 20 per cent of the parasites were obviously damaged at 2 hours.

Nucleic acid synthesis: Changes in the rate of nucleic acid synthesis were followed by injecting rats, infected from 1 to 22 days, with 0.01 mc adenine-8-C^{14} and by ascertaining the incorporation of C^{14} into the nucleic acid fraction of tryp-

anosomes 1 or 2 hours later. The data are illustrated in Figure 4. These experiments were carried out in a manner similar to the experiments with S^{35}-amino acids illustrated in Figure 1 including the initiation of infections with adult parasites. Unfortunately, no observations were made at 3 hours. On day 2, parasites which were obtained for radioassay 1 hour after giving C^{14} showed an incorporation of 392 cpm. On day 3, the 1- and 2-hour readings were 353 and 236 cpm, respectively. By day 12, these values had dropped to about 15 cpm. Thus, the values on day 12 as compared to those on day 3 represented an inhibition of 96 and 97 per cent, respectively. In neither series were the differences after day 10 statistically significant as represented by the straight lines in Figure 4. For this reason, we considered that nucleic acid synthesis was essentially inhibited by the 10th day. This conclusion is in sharp contrast to the continuing though low and decreasing synthesis of protein from day 10 through day 19.

The effect of passively transferred ablastin: Throughout the present work, we have assumed that the eventual inhibition of growth and reproduction and the associated inhibition of nucleic acid and protein synthesis in ordinary infections of the rat with *T. lewisi* are effected by ablastin. That this is true is shown by

TABLE 2

THE EFFECT OF PASSIVELY TRANSFERRED ABLASTIN ON THE INCORPORATION OF S^{35} INTO PROTEIN AND OF C^{14} INTO NUCLEIC ACIDS BY *Trypanosoma lewisi*

Ablastic serum: ml per 100 gm. rat*	Counts† per minute per 10^6 trypanosomes	
	S^{35}-amino acids (Protein)	Adenine-8-C^{14} (Nucleic acids)
2.4 to 3.5	16 ± 4 (4)‡	41 ± 8 (3)
0	47 ± 4 (4)	320 ± 27 (2)
% inhibition	66	87

* Ablastic serum was adsorbed with living *T. lewisi* to remove trypanocidal factors.
† All rats were infected with 3-day dividing trypanosomes on day 0. The passively immunized rats received ablastic serum on day 1. All rats received 0.01 mc of the isotope on day 3 and were killed 2 hours later to obtain trypanosomes for radioassay.
‡ The number of rats in each group is given in parenthesis.

initiating infections with 3-day trypanosomes and treating them 1 day later with serum containing ablastin. Three-day trypanosomes were selected for two reasons: (1) They were as far removed as practical from the spontaneous decrease in rate found in adults shortly after injection into nonimmune rats (cf. Fig. 1). (2) They were still rapidly dividing (cf. the division forms in Figs. 1, 2, and 4), but, by the next day, ablastin effects might be evident (Table 1).

For these experiments, immune serum was collected from rats in which trypanosome reproduction had been inhibited and was adsorbed with living *T. lewisi* to remove lysins and other possible trypanocidal antibodies. Thirteen experimental rats were then infected with 3-day dividing trypanosomes. One day later, seven of the rats received from 2.4 to 3.5 ml per 100 gm adsorbed ablastic immune serum while 6 controls received no serum. On day 3, four of the passively immunized rats and 4 controls were given 0.01 mc S^{35}-amino acids, and 3 of the passively immunized rats and 2 controls were given 0.01 mc adenine-8-C^{14}. Two hours after giving the isotopically labeled material, the rats were killed to obtain suitable samples of trypanosomes for radioassay. Microscopic examination of the slides made at the time the parasites were prepared for radioassay showed that those from the passively immunized rats had become adults, whereas those from rats which received no immune serum were still dividing and contained from 21 to 32

per cent division forms. Examination of Table 2 indicates that the incorporation of S^{35} was inhibited by 66 per cent and that of C^{14} by 87 per cent in the ablastin-treated animals. These marked effects produced within 48 hours by passively transferred ablastin were similar in degree to those obtained in rats which had actively acquired ablastin. Thus, the incorporation of S^{35} on day 10 was inhibited by 74 and 53 per cent of that on day 3 in the trypanosomes when assayed, respectively, 3 and 2 hours after giving the isotope (Fig. 1). Similarly, the incorporation of C^{14} on day 10 was inhibited by 96 and 95 per cent of that on day 3 when assayed, respectively, 1 and 2 hours after giving the isotope (Fig. 4).

Discussion.—A striking finding in the present work is that the highest rate of protein synthesis was observed in adult trypanosomes which had been injected into normal rats 3 hours previously. These parasites still had the morphology of adults although a few were slightly larger. Their release from ablastic action was probably the result of their dissociation from ablastin by a single washing with phosphate saline solution and their resuspension in the ablastin-free normal serum of the recipient rats. This rapid and complete dissociation following a lowering of the concentration of free uncombined antibody indicates a low avidity for ablastin (cf. Talmage[20]). Augustine[21] designated as a lag phase the time between the initiation of an infection with adults and the beginning of growth and division and found that it could last up to 2 days. He also called attention to its similarity to the bacterial lag phase. The present work substantiates his suggestion. Further work is necessary, however, to ascertain whether the increase in size of the released adults is comparable to that found in bacteria.[22]

The high metabolic rate of released adults poses a difficulty in estimating the per cent inhibition produced by ablastin. If ablastin formation causes the initial rapid fall in the rate of synthesis in infections with *T. lewisi* which are started with adult trypanosomes, the unlikely conclusion should follow that ablastin formation starts within 3 hours after the infection is initiated. If, on the other hand, as seems likely, some limiting or regulatory mechanism of the trypanosome causes the initial fall, the question arises: when does ablastin begin to act. The general activity of ablastin is best measured by division and growth of the parasites. It is not active during the first three days of infections initiated with 3-day dividing parasites[21] or on the second and third day of infections initiated with adults (Figs. 1, 2, and 4). In the present experiments, slight effects may have appeared by day 3 (Figs. 1 and 2), but no marked effect was observed before day 4 (Table 1 and Fig. 2). We have, therefore, used the rate of isotope incorporation in the 3-day trypanosomes as the zero point in ascertaining the degree of inhibition produced by ablastin. Using this measure, consistent results were obtained (1) whether the uptake of isotope *in vivo* was correlated with the disappearance of reproductive activity in the parasites as ablastin formed in ordinary infections or with the action of passively transferred ablastin, and (2) when the uptake of isotope *in vitro* was correlated with the reproductive activity of the parasites.

The rate of incorporation of S^{35} into protein and of C^{14} into nucleic acid synthesis on day 3 gave values of 58 and 236 cpm, respectively, for trypanosomes measured 2 hours after administering the isotope. These values rapidly decreased to about 15 cpm for both on day 10 as ablastin inhibited reproduction and growth of the parasites. By this time, inhibition of nucleic acid synthesis was virtually com-

plete. In contrast, a low but decreasing level of protein synthesis occurred in adult trypanosomes as indicated by the fact that protein synthesis was inhibited by 66 per cent on the 7th day and by 88 per cent on the 19th day. This low grade synthesis is probably a maintenance phenomenon in an actively motile parasite which is neither dividing nor growing. It parallels the maintenance carbohydrate metabolism described by Moulder[14] as a result of ablastic inhibition.

We have no evidence as to how ablastic inhibition of nucleic acid and protein synthesis is effected. Two possibilities are suggested by other experiments. In a study of the comparative synthesis of nucleic acid among different species of trypanosomes,[16] we found that *T. lewisi* incorporates only small amounts of C^{14} into the fraction precipitated by cold TCA from glycine-2-C^{14}. Two hours after administering 0.01 mc of the isotope, the fraction precipitated by cold TCA showed only 18 cpm and no significant part of this was in the nucleic acid portion. This result suggests that the parasite cannot synthesize the purine ring. It is possible that the antibody blocks the absorption of compounds containing the purine ring, especially adenine. The other possibility is that compounds containing the purine ring can be absorbed but that further utilization of the compounds is blocked by a derangement of carbohydrate metabolism. Such a mechanism is suggested by Moulder's[14] results indicating that ablastin changed the glucose metabolism from one of assimilation to one of maintenance. This conclusion is further suggested by our results[16] that, within 2 hours after the administration of C^{14}-glucose (uniformly labeled), dividing trypanosomes incorporated a small amount of C^{14} into the fraction precipitated by cold TCA (14 cpm), whereas adults incorporated about half this amount (8 cpm). In both the dividing and the adult parasites, approximately half of the radioactivity was in the nucleic acid portion.

The foregoing results are of especial interest since ablastin probably has the most specific reproduction-inhibiting action of any of the antibodies developed against animal parasites, as discussed in detail by Taliaferro.[2] It should be emphasized in this connection that ablastin acts in an entirely different way from the fundamentally parasiticidal immune factors which generally dispose of parasites quickly. It should not be confused with parasiticidal activities giving rise to static populations (parasitostasis) in certain protozoan infections in which the parasites remain at a constant level but continue to reproduce—frequently at an uninhibited rate.[2] Ablastin may act in a manner similar in some respects to the immune factors in helminth infections that stunt the growth and lower the reproductive capacity of the worms.

Finally, the rat–*T. lewisi* relationship is often used to illustrate successful parasitism because the parasite does not usually kill its host and the infection persists for a considerable length of time. In this successful parasitism, however, the immune response of the rat limits the metabolic activities of the parasite to such an extent that the parasite can exhibit its full synthetic abilities only for a few days or even hours in each rat host.

Summary and Conclusions.—The rate of protein synthesis in infections started with adult *Trypanosoma lewisi* was highest, during the course of infection, within a few hours after washed ablastin-inhibited parasites were placed in ablastin-free rats (Fig. 1). It decreased rapidly during the first 3 days of the infection before the appearance of appreciable amounts of ablastin. The same is probably true for

nucleic acid synthesis although our earliest observations were only made at 2 days (Fig. 4). The rates of nucleic acid and protein synthesis observed on day 3, when trypanosomes were rapidly dividing, markedly decreased thereafter as ablastin was formed. Thus, on the 10th day of the infection, nucleic acid synthesis had virtually stopped and protein synthesis was inhibited by more than 50 per cent as compared to the 3-day rate. Protein synthesis continued at a low grade for several weeks although at a gradually decreasing rate. These conclusions were reached by injecting rats once with S^{35}-amino acids or adenine-8-C^{14} from 0 to 19 or 22 days after infection and killing the rats from one to three hours later to obtain trypanosomes for radioassay. The foregoing conclusions were corroborated by experiments with passively transferred ablastin and by *in vitro* experiments.

Following a single injection of S^{35}-amino acids into rats with 3-day or with 6- to 12-day infections, the rate of protein incorporation was faster and reached a higher peak in dividing than in adults trypanosomes (Fig. 3). The loss in radioactivity was also faster, especially at first, in the dividing forms. Radioassay determinations were made from 20 minutes to 48 hours.

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† A preliminary note by W. H. Taliaferro, T. Pizzi, and P. D'Alesandro has appeared in *Science*, **127**, 1063 (1958).

‡ Inter-American Fellow of the John Simon Guggenheim Memorial Foundation. Permanent address: Departamento de Parasitologia, Universidad de Chile, Casilla 9183, Santiago de Chile.

[1] Rabinowitsch, L., and W. Kempner, *Z. Hyg. Infektionskrankh.*, **30**, 251–294 (1899).

[2] Review by Taliaferro, W. H., *Bact. Rev.*, **12**, 1–17 (1948).

[3] Taliaferro, W. H., these PROCEEDINGS, **10**, 12–16 (1924); *J. Exp. Med.*, **39**, 171–190 (1924).

[4] Taliaferro, W. H., *Amer. J. Hyg.*, **16**, 32–84 (1932); *J. Immunol.*, **35**, 303–328 (1938).

[5] Cf. Farr, R. S., *J. Infect. Dis.*, **103**, 239–262 (1958); and Taliaferro, W. H., L. G. Taliaferro, and A. Pizzi, *J. Infect. Dis.*, **105**, 197–221 (1959).

[6] Coventry, F. A., *Amer. J. Hyg.*, **12**, 336–380 (1930).

[7] Augustine, D. L., *Proc. Amer. Acad. Arts and Sci.*, **75**, 85–93 (1943).

[8] Barnes, E. A., *Iowa State Coll. J. Sci.*, **26**, 1–17 (1951).

[9] See Fig. 2 in Taliaferro, W. H., and Y. Pavlinova, *J. Parasit.*, **22**, 29–41 (1936).

[10] D'Alesandro, P. A., *J. Infect. Dis.*, **105**, 75–96 (1959).

[11] Regendanz, P., and W. Kikuth, *Zentr. Bakt. Parasitenk. Infek., Orig.*, **103**, 271–279 (1927).

[12] Thillet, C. H., Jr., and A. C. Chandler, *Science*, **125**, 346–347 (1957); and A. C. Chandler, *J. Parasit.*, **44**, 129–135 (1958).

[13] Taliaferro, W. H., and L. G. Taliaferro, *Amer. J. Hyg.*, **2**, 264–319 (1922); and Taliaferro, W. H., *J. Exp. Zool.*, **37**, 127–168 (1923).

[14] Moulder, J. W., *J. Infect. Dis.*, **83**, 42–49 (1948).

[15] Ryley, J. F., *Biol. Chem.*, **49**, 577–585 (1951); and Thurston, J. P., *Parasit.* **48**, 149–164 (1958).

[16] Pizzi, T., and W. H. Taliaferro, *J. Infect. Dis.* (in press).

[17] Moulder, J. W., *J. Infect. Dis.*, **83**, 31–41 (1948).

[18] Taliaferro, W. H., P. R. Cannon, and S. Goodloe, *Amer. J. Hyg.*, **14**, 1–37 (1931).

[19] Schneider, W. F., *J. Biol. Chem.*, **161**, 293–303 (1945).

[20] Talmage, D. W., *Ann. N. Y. Acad. Sci.*, **70**, 82–93 (1957).

[21] Augustine, D. L., *J. Infect. Dis.*, **69**, 208–214 (1941); and F. A. Coventry, *Amer. J. Hyg.*, **5**, 127–144 (1925).

[22] Oginsky, L. E., and W. W. Umbreit, *An Introduction to Bacterial Physiology*, 2d ed. (San Francisco: W. H. Freeman and Co., 1959).

THE NUCLEON-NUCLEON SPIN-ORBIT POTENTIAL*

By Gregory Breit

YALE UNIVERSITY

Communicated March 21, 1960

Introduction.—Bryan[1] has recently reported an improved fit to p-p scattering data employing a modification of the Signell-Marshak[2] potential in which the spin-orbit force is represented by a short-range potential, the effective part of which is located mainly on the outer edge of the repulsive core, somewhat as in the potential of Gammel and Thaler.[3] The fact that Bryan's fits to data have many features in common with a more extensive purely phenomenological phase-parameter analysis at Yale[4] gives increased confidence in the probable physical significance of the potential used by him. The general relationship of Bryan's central and tensor potentials to those derived by Gartenhaus[5] on the Chew–Low[6] extended source theory would further increase this confidence if there were an explanation of the spin-orbit potential.

A possibility of accounting for the general features of the $\mathbf{L} \cdot \mathbf{S}$ potential which apparently has not received attention in recent literature is that of a vector meson field. The short-range character of the potential calls for a sufficiently large meson mass. The various searches for new particles have not shown the existence of such a meson capable of strong interactions with nucleons. If it exists, it is more likely, therefore, that it is neutral rather than charged, although a sufficiently high mass could also decrease the chances of its detection even if the meson were charged. A neutral vector meson field is, however, the simplest possibility. The particles of such a field would be, in a sense, heavy photons. It has been known for some time[7] that the sign of the V_{LS} for such a field has the same relationship to its central potential as for the electromagnetic field. In order to reproduce the phenomenologically-indicated sign, it will be speculatively supposed that all nucleons carry the same heavy photon-producing charge. In the case of nucleons this charge could be taken to be a constant multiple of the baryon charge. This hypothesis accounts for the repulsion at short distances between nucleons. For the "extended source" pion-produced potential a short-range repulsion is already present in triplet-even states, but for triplet-odd states it gives an attractive potential which has customarily been modified by the phenomenological introduction of a repulsive core. Similarly, a repulsive core is helpful for the singlet-odd states even though the Gartenhaus calculation gives in this case a strongly attractive potential at short distances. The neutral heavy meson field gives a repulsive core in all states and the details of the Gartenhaus or any other pion-theoretical calculation have, on the present view, only secondary importance, since the core-producing field may be expected to modify the part of the interaction caused by pion exchange. The modification would be weak for large internucleon distances r in agreement with empirical evidence regarding the validity of the one pion exchange potential at large r. Since the nucleons do not penetrate the core appreciably and since the large meson mass makes the core potential drop off rapidly, the most effective part of V_{LS} is just outside the core. It is striking that Bryan's improved fit gave a shorter range for V_{LS} than that of the previous phenomenologi-

cal potentials. The possible origin of the nucleon-nucleon V_{LS} which has been suggested[8] does not give as steep a dependence of V_{LS} on r as Bryan's and is not likely to be more than contributory to the whole V_{LS}. As has been previously emphasized,[8] both the previously derived V_{LS} and the then attempted explanation of the core were too qualitative and did not pretend to take into account phenomena of virtual nucleon-antinucleon pair formation. On the hypothesis considered now the calculations would, furthermore, need serious modification. It appears simplest, therefore, to use the heavy meson in order to account for V_{LS} as well as the core.

Approximate Magnitudes.—In terms of the first of the references in footnote 7 the interaction potential $-J$ including the spin-orbit correction which arises from considerations of invariance to order v^2/c^2 form the combination

$$-J - \frac{3}{2}\frac{\hbar^2}{M^2c^2}\frac{dJ}{rdr}(\mathbf{L}\cdot\mathbf{S}) \tag{1}$$

for any J arising from a field with the same transformation properties as the vector field. The applicability of this form has been verified for the present purpose by the procedure of the third reference in footnote 7 for the case of special interest, viz.

$$J = -(q^2/r)e^{-\kappa r}. \tag{2}$$

Here q has the dimensions of electric charge in electrostatic units. For simplicity it is taken to have the same value for p-p, p-n, and n-n interactions. Two fits to Bryan's $V_{LS} = -12\,x^{-3}e^{-2x}$, $x = m_\pi c\,r/\hbar$ by the above formulae have been made at $x = 0.6$ with adjustment to produce a fit in $0.6 < x < 1$. In the first $\kappa = 9\,m_\pi c/\hbar$, $q^2\kappa = 4.15 \times 10^5$ Mev; in the second $\kappa = 12\,m_\pi c/\hbar$, $q^2\kappa = 2.85 \times 10^5$ Mev. The first gives a somewhat too gradual fall of V_{LS} in $0.6 < x < 1$; the second gives, on the whole, a somewhat too steep decrease but is reasonably good in reproducing the slope of log V_{LS} at $x = 0.6$. Present evidence would thus appear to indicate the mass of the vector meson to be $m_{hp} \cong 11\,m_\pi$. Here the suffix hp stands for heavy photon. Since Bryan's fits are not as good as those that have been obtained at Yale[4] by means of a gradient search employing most nucleon-nucleon scattering data, it may be expected that the V_{LS} will undergo changes in further fitting with attendant changes in m_{hp}.

For the first fit the values of the repulsive core potential $-J$ at $x = 0.5, 0.6, 0.8, 1.0$ are, respectively, 1026, 348, 43, 5.7 Mev. This core potential appears to be more than sufficiently strong. The pion potential would have to be assumed to be sufficiently strong to counter-balance the vector field in some of the region $0.6 < x < 1.0$. There appears to be no *a priori* objection to such strong pion effects, the convergence of the pion potential calculation being poor. For the second fit the core potential has values 118, 29.5, 2.0, 0.15 Mev at $x = 0.5, 0.6, 0.8, 1.0$, respectively. The core potential produces, in this case, minor effects for $x > 0.6$ but increases sufficiently steeply as x decreases below $x = 0.6$ to reproduce the main features of the phenomenological core. It is supposed that the $1/r^3$ singularity present in the OPEP is either cancelled by the as yet unknown terms of the pion potential beyond those in g^4 or else are made non-singular by nucleon structure effects [9–11] which may be expected to smear out the location of the pion source and thus to reduce the effect of the singularity. Since the hypothetical vector meson

field interacts with the nucleon field, one would expect some smearing out of the pion source function and hence a rounding off of the $1/r^3$ and $1/r^2$ potentials at small r. The large magnitude of the core potential at $x \cong 0.5$ is thus not expected to be offset by singularities at $x = 0$ of the pion-produced potential.

The proposed view regarding the core and V_{LS} appears to fit the general features of the interpretation of antinucleon-nucleon scattering by Ball and Chew[12] and the phenomenological considerations of Koba and Takeda.[13] In the work of Chew and Ball the nucleon-nucleon ($\mathfrak{N}$–$\mathfrak{N}$) core is converted into a nucleon-antinucleon ($\mathfrak{N}$–$\bar{\mathfrak{N}}$) sink within which the particles annihilate each other. This process is held responsible for the introduction of the ingoing wave boundary condition at the core radius. The vector meson field speculated on here is supposedly produced by a baryon type charge. The antinucleon constitutes a deficiency of this charge and hence the $\bar{\mathfrak{N}}$–$\mathfrak{N}$ short-range interaction may be expected to be strongly attractive. The two particles may therefore be expected to come together so as to annihilate each other. In the attractive core region some annihilations with emission of heavy photons may be expected, the simultaneous emission of two heavy mesons being expected by analogy with electromagnetic theory. A possible objection is that some of the pion-producing annihilations may be replaced by heavy vector mesons producing annihilations, while the cross section measurements[14] are concerned with the detection of pion production. The pion production has, however, an advantage in the competition on account of the direct coupling of positive to negative energy states caused by the γ_5 term in the pseudoscalar coupling and the effectiveness of heavy vector meson production is reduced by the $e^{-\kappa r}$ factor at all but the shortest $\mathfrak{N}$–$\bar{\mathfrak{N}}$ distances. The effectiveness of pion annihilation in the competition appears reasonable in the outer region of the core. If most antinucleons are annihilated in that region, it is not important whether the heavy photon process is dominant in the innermost region or not. The heavy vector mesons are, furthermore, not necessarily elementary particles. Their disintegration with pion emission is conceivable. If the mass of the heavy meson is as high as the second of the above-mentioned fits to Bryan's V_{LS} suggests, the simultaneous production of two heavy mesons would require an energy excess in the center of mass system of $2 \times 12 \times 270\, m_e c^2 - 2Mc^2 \cong 1{,}420$ Mev corresponding to roughly 3.9 Bev of incident energy in the laboratory system. The mass of the particle being in doubt, it is therefore not clear that there would have been enough energy in the experiments to make the simultaneous emission of two heavy vector mesons energetically possible. The maximum antiproton incident energy in the experiments quoted was 1.07 Bev.

Discussion.—In the calculations of Ball and Chew[12] the same V_{LS} is used for the $\mathfrak{N}$–$\bar{\mathfrak{N}}$ interaction as for $\mathfrak{N}$–$\mathfrak{N}$. On the view proposed here one would expect these interactions to differ by a sign. This circumstance does not appear to matter seriously in the existing comparison with experimental values of the total cross section because the weighted mean value of $(\mathbf{L} \cdot \mathbf{S})$ over all sublevels is zero. Ball and Chew used the Signell–Marshak V_{LS} which has a longer range than either Gammell–Thaler's or Bryan's and which does not reproduce polarization data especially well. The details of agreement with experiment obtained by Ball and Chew cannot be taken literally, therefore, and the reversal of the sign of V_{LS} for the $\mathfrak{N}$–$\bar{\mathfrak{N}}$ case does not appear unreasonable.

On the view proposed there is less reason to doubt the validity of static nucleon-nucleon potentials than heretofore. The phenomenologic core has been a way of getting around the supposedly very complicated conditions at small r. The adjustment of core radii to fit data on π–π scattering had at best a symbolic meaning, the direct effect of the core in π–π scattering theory being that of making the radial functions have nodes at the core radius. On the pion theory of nuclear forces it has been doubtful, however, that the infinitely large repulsive potential inside the core radius should be given a literal significance, it being possible to produce a node of the wave function by an attractive potential or to regard the existence of the node as a manifestation or indirect consequence of the many-body character of the problem inside the core radius. On the heavy vector meson hypothesis, however, the core has a rather literal meaning. The only region within which there is then serious doubt regarding the validity of the static potential picture is that between the region of applicability of the one pion exchange potential[15] ($x > \sim 1$) and the core. Although here also the static picture may be inadequate, it would be expected, perhaps, to be more directly applicable than inside the core. Perhaps this is one of the reasons for the good agreement[16] of calculations on the photodisintegration of the deuteron employing static potentials with experiment. It may also be connected with the success of the Brueckner method of calculating properties of nuclear matter as carried out by Gammel and Brueckner.[17] In view of changes made in the potentials obtained from fits to scattering data, the latter evidence is not conclusive and the treatment of photodisintegration data also has not been carried out with potentials representing scattering data accurately. Higher electromagnetic multipoles may also contribute to the process. Therefore, these indications must be regarded as purely qualitative.

On the viewpoint that the whole potential is essentially static, a difficulty arises in connection with the binding energy of the triton H^3. It has recently been shown by Derrick and Blatt[18] that the Gammel-Thaler potential does not give a bound state of this nucleus. As the authors remark, the reason for this failure of the Gammel–Thaler potential is the relative weakness of the central triplet even potential ($^3V_c^+$) in comparison with the tensor potential for the same state ($^3V_T^+$). According to Figure 3 of Signell and Marshak's paper,[2] the employment of the Gartenhaus potential in place of that of Gammel and Thaler does not appear obviously hopeful since $^3V_c^+$ is even smaller for the Gartenhaus potential. As long as one dealt with a core within which the two-body treatment of π–π scattering could be seriously questioned, the discrepancy with H^3 could be regarded as a manifestation of the inadequacy of the two-body picture inside the core radius. With the heavy vector meson core, however, the nucleon density inside the core is small and the effect of the core may be expected to be described rather well by a large repulsive potential, so that the discrepancy has to be taken more seriously.

It is not clear, however, that there is a discrepancy between the requirements of π–π scattering data and that of the binding energy of H^3. The reasons for this belief are as follows. (*a*) The Gammel–Thaler potential does not reproduce scattering data especially well. It represents data on polarization better than those on cross sections and from this viewpoint there is no binding reason for considering either its $^3V_c^+$ or $^3V_T^+$ to be especially accurate. (*b*) Even with the heavy meson core some velocity dependence of the potential for $x < 1$ may be expected; this is

corroborated by the qualitative features of ref. 8 which may still be expected to to apply. Arguments regarding phenomenological indications of velocity dependence have been given by Gammel and Thaler[19] and other theoretical arguments previously by Breit and Yovits.[19] (*c*) A fit similar to Bryan's has not been made to *n-p* data. It is not clear that a reasonable fit cannot be made with a potential having a larger $|^3V_c^+|$ and somewhat subordinated V_T^+. (*d*) A covariant calculation has recently been made for the g^4 term V_4 of the nucleon-nucleon potential by S. N. Gupta.[20] According to this, the $(^3V^+)_4$ potential is much deeper than the potential used by Gammel and Thaler while the $(^1V^+)_4$ agrees very well with that of Bryan's $^1V_c^+$. This agreement cannot be interpreted as indicating that the V_4 potential is adequate because V_4 is much too deep for Bryan's $^3V_c^-$ and especially because $|V_4| \gg |V_2|$ in $x_c < x < 1$ so that higher powers of g may be expected to make appreciable contributions. Nevertheless, Gupta's calculation shows that the pion interaction cannot be claimed to give a small $|^3V_c^+|$. (*e*) It is conceivable that charged heavy vector mesons participate in the interaction, that the effective core radius is thereby affected, and that the influence of $^3V_c^+$ is increased. (*f*) Even with the neutral vector meson the possibility of "tensor coupling" of the meson which has been under discussion in the theory of Møller and Rosenfeld[21] can give differences in interaction potentials which contribute both to the spin-spin interaction and to V_T. These interactions would affect the region close to the core and on account of their short range would differ qualitatively from those of the older meson theories of nuclear forces. The existence of the "tensor"-type interaction with the baryon charge might perhaps be doubted if the vector meson is an elementary particle. But if it is composite, it would not be unnatural and appears hard to exclude. In summary, the binding energy of the triton involves several unknown questions and does not appear to provide an objection to the heavy vector meson hypothesis.

It is not clear that the proposed heavy vector meson has a connection with the heavy vector meson proposed by Feynman and Gell-Mann and by Sudershan and Marshak[22] for the divergenceless current form of weak interactions theory. Since the vector meson field of very large mass is supposed to give an interaction of the proton-neutron current with itself, and since this is supposed to be "weak," the two types of heavy mesons may have only a remote relationship because the interaction used here is decidedly strong. One could still try to have the heavy vector meson postulated in this note participate in beta decay in the manner of Møller and Rosenfeld. This would leave, however, the μ decay in a different category and the universality of weak interaction coupling would be unexplained. The connection, if any, appears to be indirect.

The theory of pion-nucleon interactions experiences difficulty in accounting for the electron-neutron interaction[10] and the form factors required by electron-proton and electron-deuteron scattering experiments.[9, 11] It has proved difficult to give a reasonable charge distribution to the neutron which reconciles the theoretical requirement at large r, which arises in its supposed isovector character, with the simple phenomenologic suggestion that the neutron charge density is nearly zero while the neutron's magnetic moment form factor is the same as the proton's and also the same as that of the proton's charge distribution. The interaction with the heavy vector meson does not appear to bring in new difficulties and even appears to help in explaining this situation. If it were the only interaction present,

it would smear all nucleon electromagnetic densities by the same amounts in space. Since the "undressed" neutron has no charge, the empirically suggested picture is reproduced. The situation is, of course, complicated by the pion-nucleon interaction. Perhaps the facts should be interpreted in the sense that the heavy photon interaction is the more important of the two for nucleon structure theory.

The neutral vector version of the hypothesis correlates the large nucleon mass with the interaction with the heavy photon and the small electron mass with the interaction with the light photon. In wave field theory, the self mass on renormalization becomes the experimental mass as a consequence of the procedural conventions and the connection of the Fermion mass with properties of the Boson-field particles is lost. Nevertheless, it appears natural to have the heavier Bosons associated with the larger self-mass, whose origin is perhaps to be found partly in the energy of the virtual Bosons even though renormalization techniques imply no direct connection.

The question of the comparison of effectiveness of the vector meson field in different states has been left open throughout most of the discussion. So far as nucleon structure is concerned, the most natural variant of the theory is one in which the field acts completely symmetrically for protons and neutrons. Charge independence of nuclear forces also requires this assumption. It may be noted that according to the last reference in footnote 4 concerned with phenomenological phase shift analysis, there are indications of the correctness of charge independence not only for the one pion exchange part of the interaction but also for the part arising from smaller distances in agreement with the first indications which were based on the $^1S_0$ state. There is at present no conclusive evidence regarding the equality of spin-orbit forces or of repulsive core potentials for different values of the total isotopic spin τ. The evidence regarding the spin-orbit potential is clearer for $\tau = 1$. On the other hand, the Gammel-Thaler fits are based on a ratio of 7300/5000 of the $\tau = 1$ to the $\tau = 0$ spin-orbit potential. This number may not be regarded as final, especially in view of imperfections of fits to n-p data. There is, at present, no clear objection to supposing that this ratio will become unity with improved fits. A repulsive core has to be added to the singlet-odd Gartenhaus potential and on this basis there is again qualitative agreement with the simplest variant of the hypothesis, viz., isotopic spin independence of the interaction. On the other hand, there is no clear evidence for postulating such independence and even the possibility of participation of different meson fields comes under consideration. Further work on the interpretation of n-p scattering data and improvements in the data are desirable in this connection.

In advocating repulsive core potentials in order to preserve charge independence of nuclear forces, Jastrow[23] has mentioned the possibility that the core may be connected with the action of a heavy meson with unspecified transformation properties. At the time, however, the evidence regarding spin-orbit interactions was not clear and the repulsive core was regarded as a substitute for the proposal of Case and Pais who intended to preserve charge independence by invoking spin-orbit forces. There was no apparent reason at the time for correlating the spin-orbit potential with the core. The heavy meson proposal thus became submerged in pion-theoretic explanations of the core.

More recently S. N. Gupta[24] proposed an explanation of $\mathbf{L} \cdot \mathbf{S}$ forces in terms of

the hypothesized ρ_0 meson which has a supposed mass of roughly 2 m_π. The resulting V_{LS} agrees well with that of Signell and Marshak and of Signell, Zinn, and Marshak and is associated with an additional central attractive potential. Since the short range Gammel–Thaler and Bryan V_{LS} gives better fits to scattering data, the Signell, Zinn, and Marshak V_{LS} does not appear promising and the repulsion in the core remains unrelated to the spin-orbit potential. The correlation of the repulsion in the core with spin-orbit interaction appears, therefore, to point to the vector meson as the explanation. The possibilities of the vector meson in relating the repulsive core with the spin-orbit interaction has been entertained by the writer for some years and has been discussed with colleagues at Yale and elsewhere. The empirical evidence which has accumulated lately makes the hypothesis especially attractive and publication appears therefore desirable.

Summary.—Empirical evidence for considering the repulsive core of nucleon-nucleon interactions and the spin-orbit interaction as originating in a vector field coupling is discussed. It is shown that the large mass of the mesons which appears to fit present scattering experiments should have been an obstacle in their direct detection. Antinucleon-nucleon scattering falls in naturally with the explanation. Related phenomena such as the photodisintegration of the deuteron, the binding energy of the trition, and electromagnetic nucleon form factors are found not to contain direct contradictions with the hypothesis and even to support it to a degree.

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[1] Bryan, R. A., *Bull. Amer. Phys. Soc.*, **5**, 35 (1960), Paper IA 10. The writer is indebted to Mr. Bryan for a private discussion of his results and a preprint of a note prepared by him.

[2] Signell, P. S., and R. E. Marshak, *Phys. Rev.*, **109**, 1229 (1958); Signell, P. S., R. Zinn, and R. E. Marshak, *Phys. Rev. Letters*, **1**, 416 (1958).

[3] Gammel, J. L., and R. M. Thaler, *Phys. Rev.*, **107**, 291, 1337 (1957).

[4] Breit, G., *International Conference on Nuclear Forces and the Few Nucleon Problem*, University College, London, July, 1959. This report was based on work done in collaboration with M. H. Hull, Jr., K. D. Pyatt, Jr., C. R. Fischer, K. Lassila, and T. Degges; Hull, M. H., Jr., G. Breit, K. Lassila, K. D. Pyatt, Jr., and H. Ruppel, "Nucleon-Nucleon Phase Parameters at Energies from 9–340 Mev," paper to be read at the Washington Meeting of the American Physical Society, April, 1960; Breit, G., M. H. Hull, Jr., K. Lassila, and K. D. Pyatt, Jr., *Phys. Rev. Letters*, **4**, 79 (1960).

[5] Gartenhaus, S., *Phys. Rev.*, **107**, 291 (1957).

[6] Chew, G. F., *Phys. Rev.*, **94**, 1748 (1954); **94**, 1755 (1954); **95**, 1669 (1954); Low, F. E., *Phys. Rev.*, **97**, 1392 (1955); Chew, G. F., and F. E. Low, *Phys. Rev.*, **101**, 1570 (1956), and **101**, 1579 (1956); *cf.* also Wick, G. C., *Revs. Modern Phys.*, **27**, 339 (1955).

[7] Breit, G., *Phys. Rev.*, **51**, 248 (1937); **51**, 778 (1937); **53**, 153 (1938).

[8] *Ibid.*, **111**, 652 (1958).

[9] Hofstadter, R., F. Bumiller, and M. R. Yearian, *Revs. Modern Phys.*, **30**, 482 (1958).

[10] Foldy, L. L., *Revs. Modern Phys.*, **30**, 471 (1958).

[11] Schiff, L. I., *Revs. Modern Phys.*, **30**, 462 (1958).

[12] Ball, J., and G. F. Chew, *Phys. Rev.*, **109**, 1395 (1958).

[13] Koba, Z., and G. Takeda, *Prog. Theoret. Phys.* (Kyoto), **19**, 269 (1958).

[14] Elioff, T., L. Agnew, O. Chamberlain, H. Steiner, C. Wiegand, and T. Ypsilantis, *Phys. Rev. Letters*, **3**, 285 (1959).

[15] Breit, G., and M. H. Hull, Jr., *Nuclear Physics*, **15**, 216 (1960).

[16] Czyz, W., and J. Sawicki, *Phys. Rev.*, **110**, 900 (1958); de Swart, J. J., and R. E. Marshak, *Phys. Rev.*, **111**, 272 (1958); Nicholson, A. F., and G. E. Brown, *Bull. Amer. Phys. Soc., Ser.*

II, **3**, 172 (1958); *Proc. Phys. Soc.* (*London*), **73**, 221 (1959). Zernik, W., M. L. Rustgi, and G. Breit, *Phys. Rev.*, **114**, 1358 (1959), also report read on extension of above work by G. Breit at the International Conference on Nuclear Forces and the Few Nucleon Problem, University College, London, July, 1959.

[17] Brueckner, K. A., and J. L. Gammel, *Phys. Rev.*, **109**, 1023 (1958).

[18] Blatt, J. M., report at London International Conference on Nuclear Forces and Few Nucleon Problems, University College, London, July, 1959 (to be published). Derrick, G. H., and J. M. Blatt, *Nuclear Physics*, **8**, 310 (1958), and preprint on further work by the same authors.

[19] Gammel, J. L., and R. M. Thaler, *Annual Reviews of Nuclear Science* (to be published). Cf. Breit, G., and M. C. Yovits, *Phys. Rev.*, **81**, 416 (1951).

[20] Gupta, S. N., "Pion Theory of Nuclear Forces with Nucleon Recoil," *Phys. Rev.* (to be published). The author is grateful to Professor Gupta for an advance copy of this paper and a discussion of its contents.

[21] Møller, C., and L. Rosenfeld, *Proc. Roy. Danish Acad.*, **17**, 8, 1 (1940); Rosenfeld, L., *Nuclear Forces* (Amsterdam: North Holland Publishing Company; New York: Interscience Publishers, Inc., 1949), vol. 2.

[22] Feynman, R. P., and M. Gell-Mann, *Phys. Rev.*, **109**, 193 (1958); Sudershan, E. C. G., and R. E. Marshak, *Phys. Rev.*, **109**, 1860 (1958).

[23] Jastrow, R., *Phys. Rev.*, **79**, 389 (1950).

[24] Gupta, S. N., *Phys. Rev. Letters*, **2**, 124 (1959).

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*STUDIES IN A TEMPORAL CLASSIFICATION OF REINFORCEMENT SCHEDULES: SUMMARY AND PROJECTION**

BY W. N. SCHOENFELD AND W. W. CUMMING

COLUMBIA UNIVERSITY

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The present paper reports a new research, and places it in the context of related studies arising from an earlier suggestion regarding a system of temporal variables for the classification of reinforcement schedules.[1] That system defines an interval, t^D, during which a specified instrumental response of the organism may be followed by a reinforcing event, and an interval, t^Δ, during which the specified response is not followed by a reinforcing event. The fundamental cases are established with three restrictions upon the ways in which t^D and t^Δ are used: (1) t^D and t^Δ are held constant, (2) t^D and t^Δ are alternated, and (3) only the first response in t^D is reinforced. Given these defining properties, two variables suggest themselves. The first of these is the interval $t^D + t^\Delta$, or cycle length; the second is the proportion of the cycle length during which a response may be reinforced, $t^D/(t^D + t^\Delta)$, or $\bar{T}$. Variations in schedule may be set up along a number of lines by altering the three restrictions mentioned here, and in other ways suggested in the earlier paper.

A number of studies[2-5] have been carried out attempting to map the domain suggested by the variables of cycle length and $\bar{T}$. The new study reported here, involving a cycle length of 1,800 seconds (30 minutes) with $\bar{T}$ varying between 1.00 and 0.00025, adds an additional function to those already obtained. The present data may also be examined in conjunction with those of a study,[6] preparatory to the present one, in which cycle length was 30 minutes but $\bar{T}$ kept invariant at 1.00.

TABLE 1

RESPONSE RATES (CORRECTED) AND RESPONSES PER REINFORCEMENT (R's/S^R), FOR EACH BIRD ON THE SCHEDULE ($t^D + t^\Delta$ = 1,800 SEC, $\bar{T}$ VARIANT) AVERAGED FOR THE LAST TWELVE DAYS OF EXPOSURE TO EACH $\bar{T}$

$\bar{T}$ =	1.00	0.067	0.033	0.017	0.0083	0.0042	0.0021	0.0010	0.0005	0.00025
t^D(sec) =	1,800	120	60	30	15	7.5	3.75	1.88	0.94	0.47
Bird No. 62										
Rate (resp./min.)	24.42	30.52	19.04	26.59	19.21	21.22	35.47	26.00	14.86	11.88
R's/S^R	732.53	1,167.29	1,866.44	1,741.52	2,050.81	1,525.66	1,386.32	1,599.06	2,792.30	3,111.39
Bird No. 64										
Rate (resp./min.)	15.58	3.14	11.76	21.35	8.51	5.63	16.43	1.49	0.002	0
R's/S^R	465.83	221.61	794.49	1,155.30	909.32	958.71	1,547.07	1,723.00	...	...
Bird No. 68										
Rate (resp./min.)	8.08	18.31	24.20	17.32	18.23	14.69	13.41	3.84	0.22	.07
R's/S^R	242.18	569.49	1,050.21	635.57	1,107.03	766.27	891.85	1,433.87	2,533.00	...
Mean Rate	16.02	17.32	18.33	21.75	15.32	13.85	21.77	10.44	5.02	...
Mean R's/S^R	480.18	652.80	1,237.05	1,177.46	1,355.72	1,083.55	1,275.08	1,585.31	2,662.65	...

Birds No. 64 and No. 68 had essentially extinguished on $\bar{T}$ of 0.0005, but a rate average for the three birds was taken nevertheless and plotted in Figure 1; no average was taken or plotted for $\bar{T}$ of 0.00025. As stated in the text, Bird No. 62 received no reinforcements in the first twelve days on schedule $\bar{T}$ of 0.00012, and while responding was in evidence, t was sporadic and extinguishing. The absence of R's/S^R entries in some of the extreme cells indicates that no reinforcements were obtained in the last twelve days on that schedule.

TABLE 2

CORRECTED RESPONSE RATES (RESP./MIN.) IN ALL STUDIES USING THE TWO TEMPORAL VARIABLES ($t^D + t^\Delta$) AND $\bar{T}$

$(t^D + t^\Delta)$										
0.001 sec (ref. 3)	$\bar{T}$	1.00	0.100	0.020	0.010	0.005	0.0025			
	Mean rate	27.3	172.44	213.90	202.68	183.48	153.78			
30 sec (ref. 4)	$\bar{T}$	1.00	0.35	0.05	0.03	0.02	0.013			
	Mean rate	41.8	48.8	62.1	69.6	87.4	107.3			
120 sec (ref. 5)	$\bar{T}$	1.00	0.05	0.025	0.016	0.008	0.004	0.003	0.002	
	Mean rate	35.6	36.5	36.7	41.7	55.3	66.1	78.4	95.4	
600 sec (ref. 5)	$\bar{T}$	1.00	0.0083	0.0066	0.0050	0.0033	0.0016			
	Mean rate	23.2	22.4	28.3	37.8	57.4	47.1			
1,800 sec (present study)	$\bar{T}$	1.00	0.067	0.033	0.017	0.0083	0.0042	0.0021	0.0010	0.0005
	Mean rate	16.02	17.32	18.33	21.75	15.32	13.85	21.77	10.44	5.02
$\frac{\bar{T}}{0.05^{(2)}}$	Cycle Length (sec)	30	15	7.5	3.75	1.88	0.94			
	Mean rate	27.8	50.7	95.6	145.1	160.6	187.7			

Method.—With few exceptions, the apparatus, subjects, and procedure were identical to those used in the earlier studies. The subjects were four white Carneaux hen pigeons, but data are reported here on only three of these as one died in mid-experiment. The subjects were exposed to the reinforcement schedules daily for sixteen consecutive hours, starting at approximately 5:00 P.M. and continuing until approximately 9:00 A.M. the next morning.

In earlier studies, subjects were given daily exposures to the reinforcement schedule until their behavior met a specially contrived "stability criterion." For reasons cited elsewhere,[7] this criterion was not employed in this study. Instead, animals were exposed to each schedule for thirty consecutive days and were then changed to the next value of $\bar{T}$. The values of $\bar{T}$ used in this study, in the order in which they were presented, were 1.00, 0.067, 0.033, 0.017, 0.0083, 0.0042, 0.0021, 0.0010, 0.0005, and 0.00025, corresponding to t^D periods within the half hour cycle length of 1,800, 120, 60, 30, 15, 7.5, 3.75, 1.88, 0.94, and 0.47 seconds, respectively.

Results and Discussion.—The last twelve days of exposure to each schedule were used for the computations presented in Table 1. The table contains, for each schedule and for each of the three subjects completing the experiment, mean rate in responses per minute (corrected as usual for the total amount of time of reinforcement, i.e., food hopper presentation), and the mean number of responses per reinforcement. For Birds Nos. 64 and 68, extinction to a zero rate occurred when presented with the 0.47 second t^D; Bird 62, after moving to the 0.24 second schedule, did not receive a single reinforcement in its first twelve days on that schedule, and so the experiment was terminated although the animal was still responding sporadically.

The function relating rate to $\bar{T}$ is essentially flat, except for a drop in rate at the smallest values of $\bar{T}$. The general flatness of the function over a wide range of $\bar{T}$ is noteworthy, since the "missing" of reinforcements began at the $\bar{T}$ of 0.067 though it did not become marked until smaller values of $\bar{T}$ in the vicinity of $\bar{T}$ of 0.033 to 0.0083. The inversion in the function at $\bar{T} = 0.0021$ may be related to behavioral changes involving response "breaks" after reinforcement which shorten conspicuously around this value of $\bar{T}$, producing an over-all higher rate. Evidence for this, and an examination of other details of these data, will be offered elsewhere at a later time.

It should be noted that the function relating responses per reinforcement to $\bar{T}$ shows a general rise as the value of $\bar{T}$ decreases. At small values of $\bar{T}$, it is possible to maintain behavior even though only one out of every 2,000 responses or more is followed by reinforcement.

It seems useful at this time to collect the functions obtained thus far with the temporal variables mentioned earlier. Table 2 summarizes six functions within the experimental domain. Five of these functions,[3–5] including the present one, held cycle length constant while varying $\bar{T}$. A sixth function[2] held $\bar{T}$ constant while varying cycle length. These six functions are shown in graphical form in Figure 1, and suggest a surface which may be related to the schematic domain outlined by Schoenfeld, Cumming, and Hearst.[1] For reference, that schema is included as an inset in Figure 1, its sectors representing suggested correlates with classically defined schedules. Thus, sector B corresponds roughly with "regular reinforcement," sector E with "random ratio reinforcement," sector A with "fixed

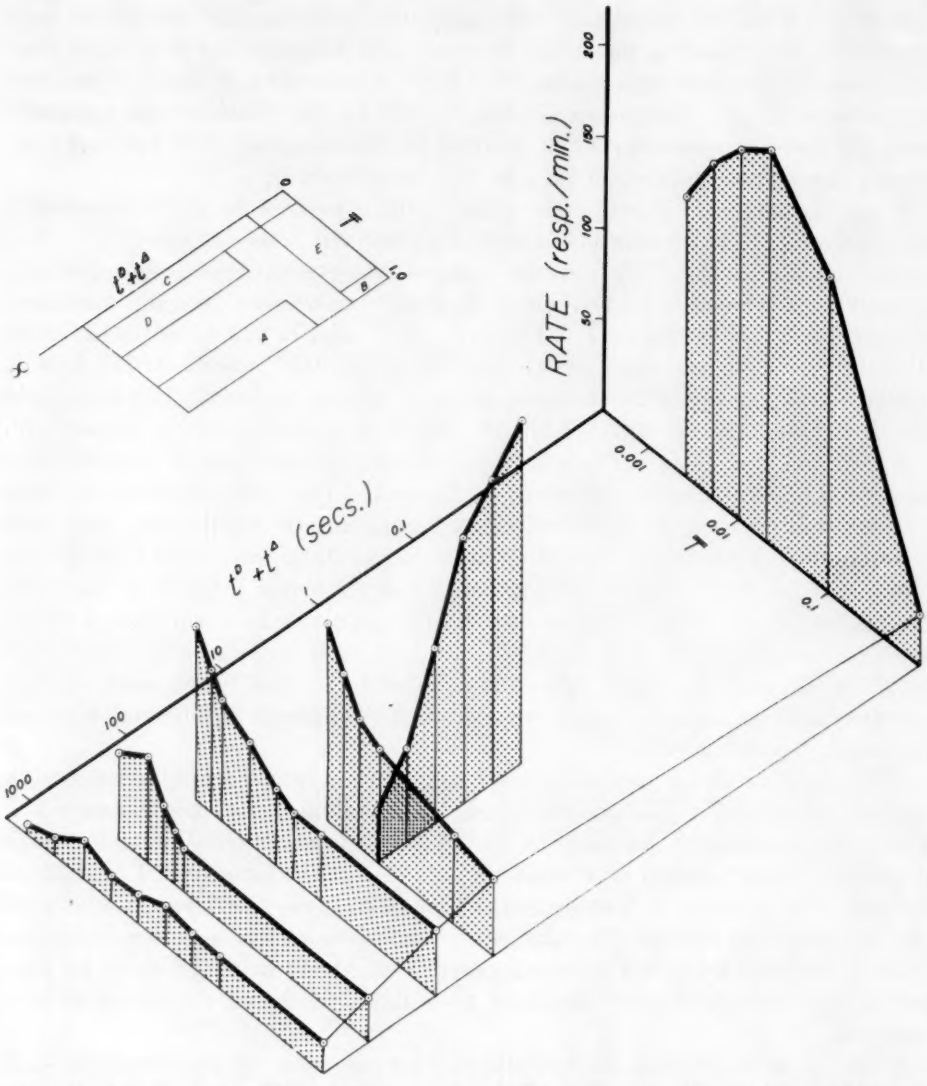


FIG. 1.—Summary of studies relating response rate to the two systematic variables, cycle length and $\bar{T}$. The $t^D + t^\Delta$ and $\bar{T}$ scales are logarithmic, while the rate ordinate is arithmetic; exact numerical values are found in Table 2. The inset shows how the experimental domain may be related to some classically defined reinforcement schedules, as suggested by Schoenfeld, Cumming, and Hearst.[1]

interval reinforcement," sector D with "fixed ratio reinforcement," and sector C with "extinction." Temporal schedules corresponding roughly with "random ratio" schedules are shown to produce very high rates of responding with a maximum near $\bar{T} = 0.02$. A long ridge of high response rates runs through this terrain in the area of sector D, while a shallow plain characterizes sector A. Relatively low rates of responding are found, not only at very long cycle lengths, but also at those very short cycle lengths corresponding to regular reinforcement.

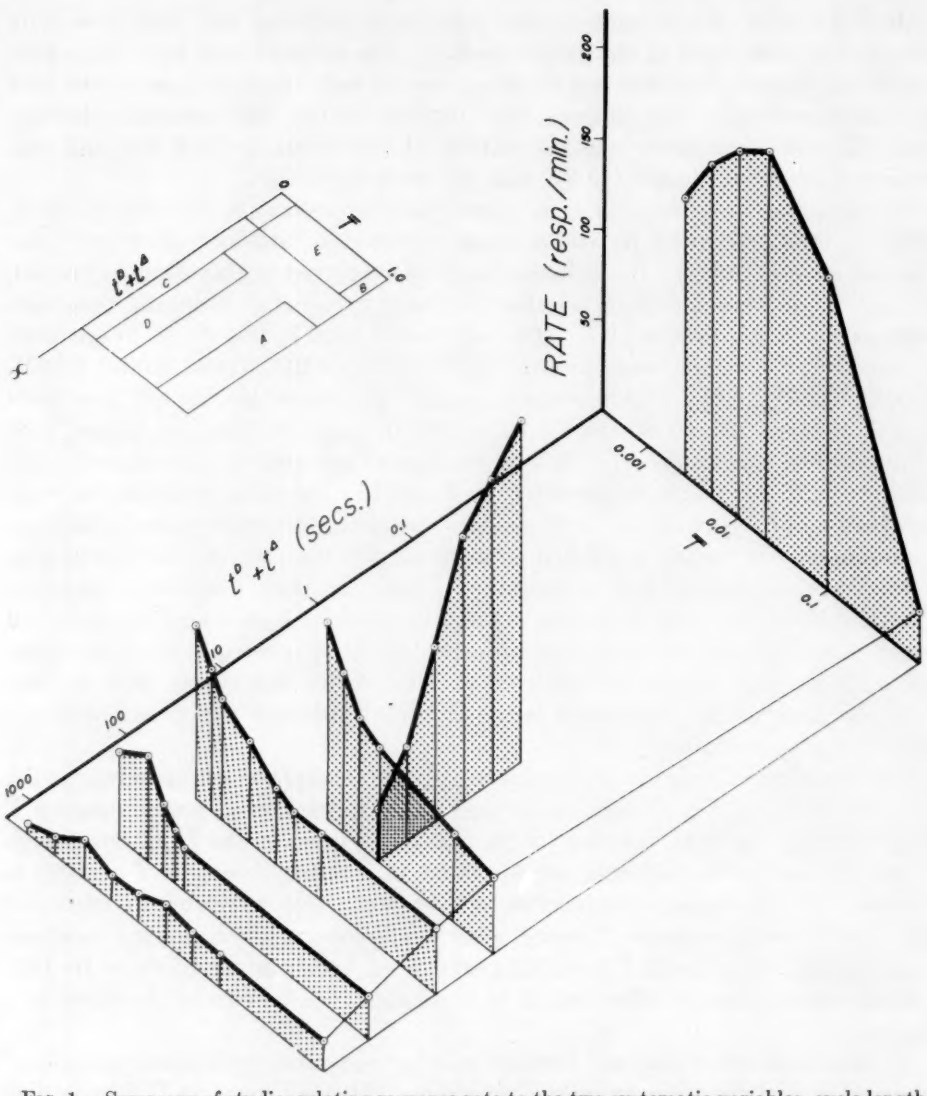
It may be recognized that it would probably not be useful to pursue the surface

beyond a cycle length of 30 minutes, because even at this value the "breaks" after reinforcement are so large as to make somewhat dubious an average response rate taken over the whole of any experimental session, and, indeed, this problem exists with each of the functions in our surface. Nevertheless, we have here discussed rates without regard to this question partly because the definition of "break" is not unambiguous and partly to preserve consistency with the treatment of earlier functions.

The Tau System.—Concentrating exclusively upon a response as an event in time (ignoring its other dimensions), and considering only single-valued reinforcement contingencies, classical reinforcement schedules were defined either in terms of a time variable ("interval" schedules) or a response count variable ("ratio" schedules), or some combination of the two. This duality had face validity, so to speak, both because of the irreducible metrics of time and count, and because of the different behavioral effects produced by the two types of schedule. Nevertheless, the t^D, t^Δ system of temporal classification of reinforcement schedules, in addition to its utility and interest apart from any subsumption of classical schedules, attempted to sidestep that duality. It did so by arguing that "ratio-type" behavior arises from any reinforcement procedure which increases response rate, that therefore the count specification of a schedule is irrelevant; that a proper selection of t^D, t^Δ values alone would generate high response rates and attendant "ratio-type" behavior, and that "ratio" schedules could therefore be regarded as special or limiting cases of temporally defined ones (those producing constant response rates and relatively constant "breaks" after reinforcement, so that a fixed, or controllably variable, number of responses would occur prior to reinforcement availability). Empirical studies to date, testing these ideas, have met with some measure of success, and encourage further exploration of the t^D, t^Δ system and its parameters.[8]

Despite this success, however, one basic feature of the t^D, t^Δ system seemed to call for refinement, namely, the fact that the t^D, t^Δ framework yet contaminates the independent and dependent variables of experiments conducted within the system (as do the classically defined schedules). The contamination takes the form of specifying the independent variable by reference to the behavior of the organism, or dependent variable; thus, with the primitive restriction that only the first response in t^D be reinforced, the effective length of t^D and the onset of t^Δ in any cycle are determined by the organism rather than the experimenter. In some senses, this problem may be viewed as merely definitional within the t^D, t^Δ system, but this does not obscure the fact that the animal's behavior affects the reinforcement contingency. One consequence of this contamination, again under the rule of the same primitive restriction, is that it is possible in the t^D, t^Δ system to define a schedule formally equivalent to the classical "fixed interval" by setting $\bar{T}$, or $t^D/(t^D + t^\Delta)$, equal to 1.00, when the probability of reinforcement for the first response in t^D is also equal to unity.

All that is required to eliminate the contamination problem, is to regress one step in the temporal level of the classificatory system. Using a supplementary set of terms, we can define a new schedule composed of cycles ($\tau^D + \tau^\Delta$) during a part of which (τ^D) reinforcement is available, and during the other part of which (τ^Δ) reinforcement is not available (or less probable). With respect to the whole



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All that is required to eliminate the contamination problem, is to regress one step in the temporal level of the classificatory system. Using a supplementary set of terms, we can define a new schedule composed of cycles ($\tau^D + \tau^\Delta$) during a part of which (τ^D) reinforcement is available, and during the other part of which (τ^Δ) reinforcement is not available (or less probable). With respect to the whole

cycle, the ratio $\tau^D/(\tau^D + \tau^\Delta)$, or $\bar{\tau}$, can take any value between zero and unity, and the usual parametric and restrictive considerations apply to the *tau* variables as to the t variables (e.g., the parameter of probability of reinforcement in τ^D and τ^Δ; type of alternation of τ^D and τ^Δ; fixed or varying lengths of τ^D and τ^Δ in any one schedule; and so on), with the exception that the restriction is lifted that only the first (or, any ordinally specified) response in τ^D is reinforced. Within τ^D, a t^D, t^Δ schedule is in force with all the parametric possibilities that are usual in the t system; for this t^D,t^Δ schedule, the restriction is lifted that only the first response in t^D is reinforced, and, moreover, cycle lengths are kept very short with respect to minimal inter-response time so that probability of reinforcement is (*a*) equal for all responses in τ^D, (*b*) independent of inter-response time, and (*c*) expressible by the ratio $\bar{T}$. Where the probability of reinforcement in τ^Δ is desired to be above zero (but necessarily still below that in τ^D), the t^D, t^Δ schedule in force during τ^Δ would accordingly be set so as to yield a lower reinforcement probability (e.g., by a smaller $\bar{T}$). Since all responses occurring in τ^D which coincide with any t^D are reinforced, no contamination of the experimental independent and dependent variables exist. One mark of this evolution is the fact that no reinforcement schedule is definable in the *iau* system that is formally equivalent to a classical "interval" schedule. The only equivalences remaining are to the basic operant contingencies of regular reinforcement ($\bar{\tau} = \bar{T} = 1.00$), and of extinction ($\bar{\tau} = \bar{T} =$ zero), so that any remaining argument regarding contamination is transferred to another and prior theoretical level (namely, the nature of the "operant" class of conditioning procedure). It is evident that the *tau* system represents the first step in a potentially infinite regress, but to accomplish the purposes set forth no more than this one step is needed.

* This research was supported by the National Science Foundation under Grants G-3408, G-5517, and G-8671.

[1] Schoenfeld, W. N., W. W. Cumming, and E. Hearst, "On the Classification of Reinforcement Schedules," these PROCEEDINGS, **42**, 563–570 (1956).

[2] Schoenfeld, W. N., and W. W. Cumming, "Some Effects of Alternation Rate in a Time-Correlated Reinforcement Contingency," these PROCEEDINGS, **43**, 349–354 (1957).

[3] Brandauer, C. M., "The Effects of Uniform Probabilities of Reinforcement upon the Response Rate of the Pigeon," unpublished Ph.D. dissertation, Columbia University, 1958.

[4] Hearst, E. "The Behavioral Effects of Some Temporally Defined Schedules of Reinforcement," unpublished Ph.D. dissertation, Columbia University, 1956. An abbreviated report based on this dissertation appears in *J. Exptl. Anal. Behav.*, **1**, 45–56 (1958).

[5] Clark, R., "The Behavioral Effects of Some Time-Correlated Reinforcement Schedules," unpublished Ph.D. dissertation, Columbia University, 1958. An abbreviated report based on this dissertation appears in *J. Exptl. Anal. Behav.*, **2**, 1–22 (1959).

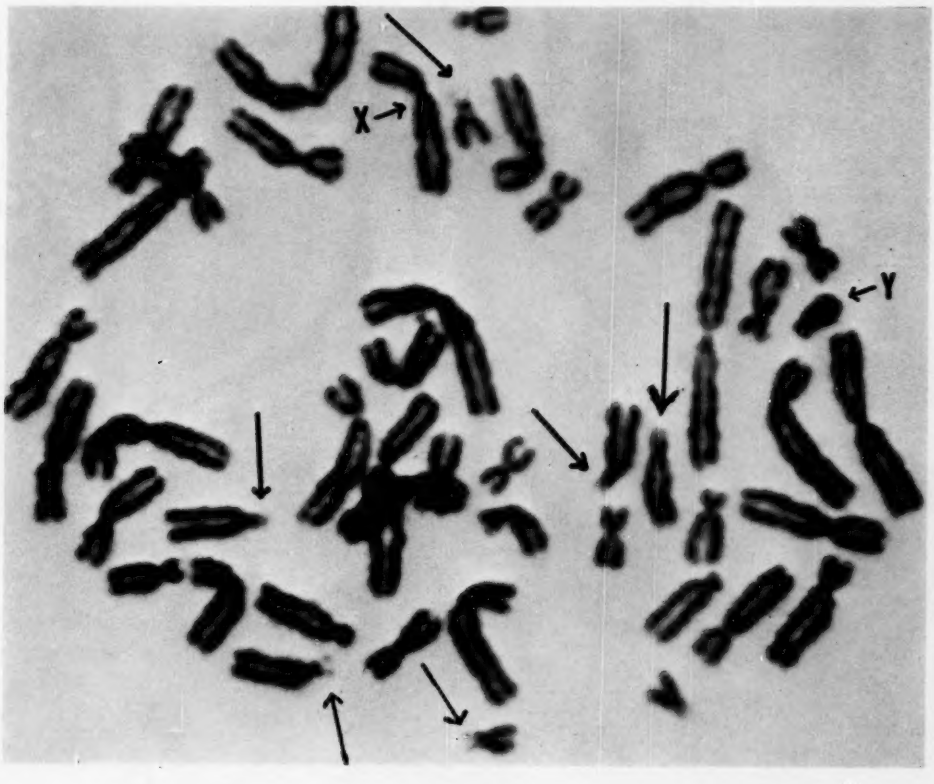
[6] Cumming, W. W., and W. N. Schoenfeld, "Behavior under Extended Exposure to a High-Value Fixed Interval Reinforcement Schedule," *J. Exptl. Anal. Behav.*, **1**, 245–263 (1958).

[7] Cumming, W. W., and W. N. Schoenfeld, "Behavior Stability under Extended Exposure to a Time-Correlated Reinforcement Contingency," *J. Exptl. Anal. Behav.*, **3**, 71–82, (1960).

[8] Cf. Millenson, J. R., "Some Behavioral Effects of a Randomly Alternating Two-Valued Temporally Defined Schedule of Reinforcement," unpublished Ph.D. dissertation, Columbia University, 1959. An abbreviated report based on this dissertation appears in *J. Exptl. Anal. Behav.*, **2**, 191–202 (1959).

ERRATUM

This photograph replaces that appearing on page 534 of these PROCEEDINGS, **46** (1960), as figure 2 in the paper by Tjio, Puck, and Robinson, entitled "The Human Chromosomal Satellites in Normal Persons and in Two Patients with Marfan's Syndrome." The original photograph contained a misplaced arrow, failed to show clearly the 6 chromosomal satellites, and did not reveal the identities of the X and Y chromosomes.



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